THIAMINE DEFICIENCY IN THE CALF

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THREE FIGURES

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A synthetic milk diet which is satisfactory for the nutrition of the young calf has been reported (Wiese et al., '47a). Using this diet it has been found that nicotinic acid is not required by the newborn calf (Johnson et al., '47), while biotin and riboflavin deficiencies can be produced (Wiese et al., '46, '47b). In this paper we wish to report the results of the elimination of thiamine from the diet of the young dairy calf.

EXPERIMENTAL

The composition of the thiamine-deficient "synthetic milk" ration fed is given in table 1. Ascorbic acid and nicotinic acid have been omitted, since previous work has demonstrated that they are not required (Wiese et al., '47a; Johnson et al., '47).

The experimental animals on this thiamine-free ration were male calves (5 Holsteins, 1 Jersey and 1 Red Poll-Jersey cross), cared for as previously reported (Wiese et al., '47a).

During the same experimental period, 7 other male calves (3 Holsteins, 3 Guernseys and 1 Jersey) were raised on the same diet with the addition of 0.65 mg of thiamine per kilogram of liquid ration and these animals served as simultaneous positive controls. The growth curves of the calves on this com-

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plete ration are given in figure 1. In general, the growth rates of these controls are similar to those of positive controls previously reported from this laboratory (Johnson et al., '47), and quite similar to the Ragsdale ('44) normals. Jersey calf 33 twice suffered from respiratory infections — once during the first week, and again during the tenth and eleventh weeks of feeding. Both times the animal responded to sulfamerazine treatment, but its net gain was lower than normal, due to these 2 setbacks.

TABLE 1

Composition of synthetic milk.

COMPONENTS	PER CENT	VITAMINS IN MG PER KG OF LIQUID "MILK." ADDED AT TIME OF FEEDING	
Casein (Labco)	30.0	Riboflavin	1.3
Lard	26.6	Pyridoxine	0.65
Salt mixture	4.0	Calcium pantothenate	2.6
Cerelose	39.4	α-Tocopherol acetate [1]	1.0
		2-Methyl-1,4-naphthoquinone	0.26
		Inositol	26.0
		Choline	260.0
		p-Aminobenzoic acid	2.6
		Pteroylglutamic acid	0.052
		Biotin	0.01
Vitamin A — 5,000 I.U. per day			
Vitamin D — 500 I.U. per day			

[1] The α-tocopherol acetate and vitamin K dissolved in a small amount of lard, were homogenized along with the rest of the lard into the solution of casein, salts and cerelose.

The growth curves of the 7 calves on the thiamine-deficient ration are given in figure 2.

Two general types of symptoms were shown by the calves on the thiamine-low ration; one group (calves 27, 31, 32 and 39) showed acute symptoms, dying before a cure could be obtained; and the other group (calves 16, 17 and 36) showed a more chronic, and thus more strikingly characteristic, thiamine-deficiency syndrome. It is possible that calves 16 and 17 had better body stores of thiamine while calf 36 received

thiamine in its diet for the first 10 days. This may explain the fact that these animals were slower in coming down with the deficiency and showed more characteristic symptoms.

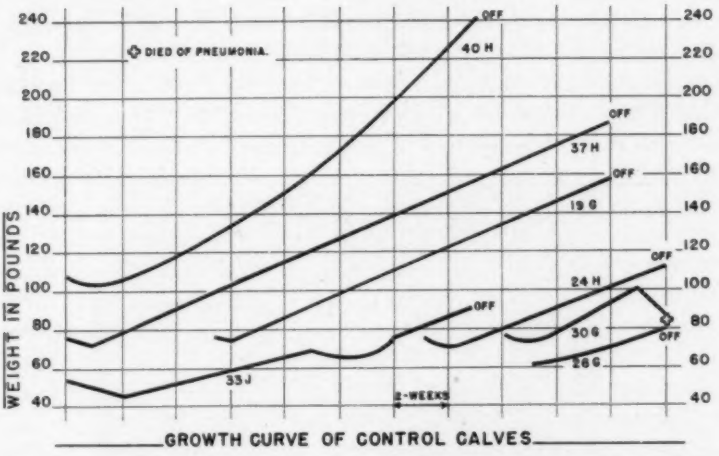

Fig. 1 Growth curves of control calves fed the complete thiamine-containing ration.

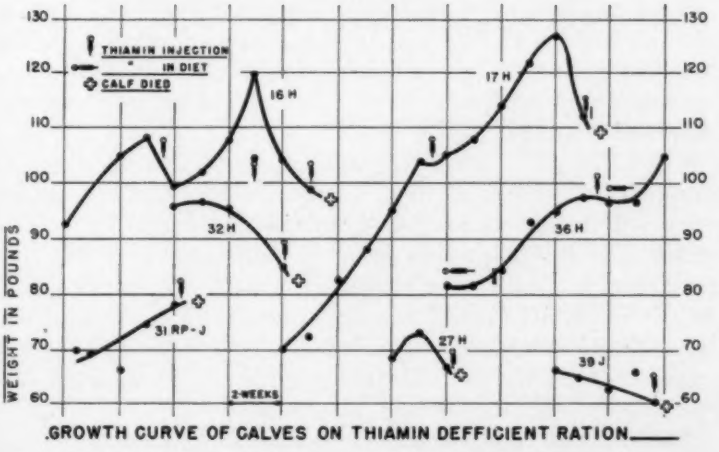

Fig. 2 Growth curves of calves fed the thiamine-low protein.

The acute syndrome consists of practically complete anorexia for 1 or 2 days, during which time there is very severe scouring, resulting within 24 hours in severe dehydration, collapsed veins and death. We have been unable to cure any of these severely dehydrated animals by intravenous injection of thiamine or thiamine plus 10% glucose solution.

Calves 16, 17 and 36, however, showed much more specific symptoms. These consisted of some anorexia, which appeared several days before any further symptoms, and was followed by the appearance of some mucous discharge from mouth and eyes, which was not as severe or prolonged as in the case of riboflavin deficiency. Later, poor coordination of the legs and marked weakness occurred. The animals were unable to stand, and when placed on their feet, their legs would spread out sideways, and the calf, having no control over them, would fall down. Two calves (17 and 36) had convulsions, while calf 16 showed constant muscle twitching and trembling. Some diarrhea occurred in these animals, but not as severe as in the first group, and there was little dehydration.

The response to the injection of 10 mg of thiamine in 10 ml normal saline, given intravenously, was almost miraculous in these 3 calves. The twitching and shaking stopped in 15 minutes, and within an hour calf 16 was able to eat. Calf 36, which had had 2 convulsions within one-half hour, was able to get up and stand normally within an hour after receiving the thiamine injection.

Calves 16 and 17 were given this 1 injection of 10 mg of thiamine at the time of their first severe symptoms, and then were not further treated until in the last stages of relapse which occurred 4–5 weeks later in both cases. At this time both calves showed the characteristic acute symptoms shown by calves 27, 31, 32 and 39, consisting of anorexia, scouring, severe weight loss, and dehydration. The injection of thiamine at this stage did not prevent the death of calves 16 and 17.

The fact that calves 16 and 17 responded to thiamine therapy and then later died during a relapse of symptoms which were

apparently the same as those shown by calves 27, 31, 32 and 39, is confirmatory evidence that the acute syndrome seen in these latter calves was in fact due to the absence of thiamine from the diet. This is further substantiated by the positive control animals, none of which showed these symptoms.

Calf 36 continued to receive thiamine in its diet after the initial injection, and this calf made an uneventful recovery.

Figure 3 pictures calf 17 when first deficient, showing the poor leg coordination and inability to stand alone.

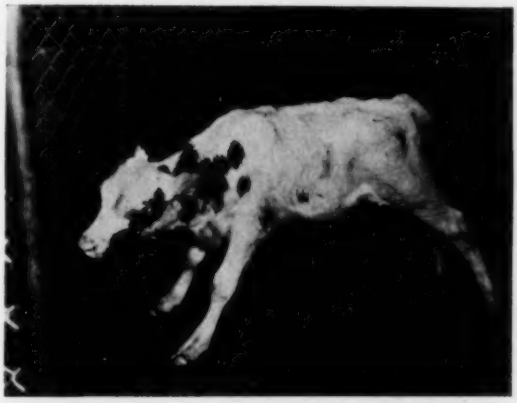

Fig. 3 Calf 17 — showing first symptoms of thiamine deficiency.

In general, 24-hour urine samples were collected from each calf once a week. These samples were analyzed for thiamine by the thiochrome procedure of Mickelsen, Cardiff and Keys ('45) and for pyruvate by the procedure of Li and Kato ('41). The urinary excretions of thiamine by the deficient calves and by some of the positive control calves are given in table 2.

Fasting post-absorptive blood samples were taken from the jugular vein each week and were analyzed for thiamine and for pyruvate. Urinary pyruvate excretion was also determined, and the blood and urine values obtained are given in table 3. In the case of the deficient calves, there are given wherever possible the last values obtained before symp-

TABLE 2

Urinary excretion of thiamine in μg per day.

WEEKS ON DIET	REPRESENTATIVE POSITIVE CONTROL CALVES						CALVES ON THIAMINE-DEFICIENT RATION					
	Approx. intake	24	26	30	33	40	16	17	31	32	36	39
	mg day	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*
		no colostrum										
0	..	384	116	190	69	18	158	217	249	180	..	77
1	2	226	71	63	96	80	40	50	25	62	46	21
2	2.7	...	146	104	115	173	20	14	2	17	14	40
3	3.3	187	203	114	...	413	11	0	x dead	4	14	4
4	3.6 *	...		101	...	353	3 x 18	8		x dead	8	x dead
5	3.9	197		104	283		7	5 x 300			... x *	
6	4.2	155		dead	...		5 x	22			* *	
7	4.4				...		25	22 F			130	
8	4.6				...		..	58 F			recovered	
9	4.8				124		8 x	34 F				
10							dead	69 F				
11								x dead				

x One thiamine injection given during this week.

F During this period calf 17 scoured a great deal and these high values are probably a reflection of fecal contamination which was not entirely prevented by the feces bags used.

* Thiamine given orally at each feeding this week.

toms set in and those obtained after the animals were cured. In the case of the control calves, average values are given.

In all cases of scouring, the first treatment used was oral sulfathalidine, and whenever there was any evidence of respiratory disturbance, sulfamerazine was given. Neither of these treatments had any apparent effect on the course of the deficiency, although they have been effective in correcting the disturbances for which they were given in cases involving the positive control calves.

TABLE 3

Blood and urine pyruvate on calves fed thiamine-low rations as compared to calves on the control ration.

CALF NO.	URINARY PYRUVATE, MG/DAY AS PYRUVIC ACID		BLOOD PYRUVATE, MG/ML AS PYRUVIC ACID	
	Deficient calves			
	Before thiamine injection	*After thiamine injection*	*Before thiamine injection*	*After thiamine injection*
16	107	44	..	..
17	95	39	67	25
31	65	died	20[1]	..
32	46[1]	died	24[1]	..
36	75	32	45	21
39	64	died	24	..
	Positive control calves (average values)			
24	43		26	
26	27		22	
30	32		19	
33	23		19	
40	34		21	

[1] Average values.

SUMMARY AND CONCLUSIONS

Fourteen new-born dairy calves were used in a study of their thiamine requirements. Thirteen of these had colostrum for from 12 to 48 hours and one had no colostrum. All received the highly purified diet previously used in these studies. Seven of the calves served as positive controls and 7 as test animals; the only difference in the treatment of the 2 groups was that

• Thiamine given orally at each feeding this week.

the positive controls received thiamine, whereas the test group did not. Records of daily observations for signs of deficiency, weekly weights, and weekly examination of 24-hour urine samples and of blood for thiamine and pyruvic acid were made for 11 weeks, or for as long as the calves lived. From the data obtained, the following conclusions seem justified:

1. The bovine species requires thiamine, supplied either in the diet or by the symbiotic action of rumen or intestinal microorganisms.

2. On a thiamine-low diet the calf develops pathological signs, urinary excretion usually reaches a level below 10 μg per day, and blood and urinary pyruvate levels are elevated above normal.

3. Thiamine deficiency in the young dairy calf is characterized by weakness, incoordination of legs, convulsions and head retraction, and, in some calves, by severe scouring, anorexia and dehydration.

4. The convulsions and typical polyneuritic symptoms respond almost immediately to thiamine therapy. However, in all cases of severe dehydration, the calves died, even though thiamine was administered.

ACKNOWLEDGMENTS

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Sulfathalidine (phthalylsulfathiazole) was generously supplied by Sharpe and Dohme, Philadelphia, Pennsylvania, through the courtesy of Dr. S. F. Scheidy.

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UNIDENTIFIED FACTORS ESSENTIAL FOR GROWTH AND HEMOGLOBIN PRODUCTION IN FOXES[1]

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TWO FIGURES

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INTRODUCTION

In our original studies on the nutritional requirements of the fox, the importance of folic acid was clearly established (Schaefer et al., '47), and preliminary results indicated that fresh liver contains an unknown factor essential for this species. When fox pups or adults were fed a purified ration containing all the known crystalline vitamins, including folic acid, adult animals maintained body weight for 32 to 40 weeks and weanling animals gained weight for 15 to 20 weeks, before showing general failure and sudden death. The deficiency syndrome could be alleviated or prevented by supplementing the ration with fresh raw liver or raw whole milk. Further evidence that foxes require an unidentified factor (or factors), which is distinct from the known crystalline vitamins, is presented here.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was reported briefly in Fed. Proc., *6:* 420, 1947. These studies were supported by project 614, Wisconsin Agricultural Experiment Station.

EXPERIMENTAL

Five adult silver foxes and 15 silver, platinum silver, and red fox pups from 9 different litters were used in this experiment. The basal ration had the following percentage composition: sucrose 66, casein [2] 19, cottonseed oil 8, cod liver oil 3 and salts IV (Phillips and Hart, '35) 4. Each 100 gm of ration was supplemented with 0.2 mg thiamine chloride, 0.2 mg pyridoxine hydrochloride, 0.4 mg riboflavin, 1.5 mg calcium pantothenate, 4.0 mg niacin, 100 mg choline chloride, 0.1 mg folic acid, 0.025 mg biotin, 25 mg i-inositol, 50 mg p-aminobenzoic acid, 2 mg α-tocopherol and 0.5 mg 2-methyl-1,4-naphthoquinone. The general experimental procedures were the same as those reported previously (Schaefer et al., '47). When the animals showed deficiency symptoms indicated by a 15 to 30% loss in body weight, reduction in hemoglobin, depigmented matted fur, anorexia and in some cases paralysis, they were fed the supplements shown in table 1. If an animal failed to show a response after administration of the supplement for 4 weeks, the material as fed was considered to be inactive. In some animals failure was rapid, and where death appeared imminent it was necessary to administer additional therapy prior to completion of the 4-week period.

Although variations in red and white blood cell counts were observed, no particular trend was apparent. At the time of the deficiency just prior to administration of therapy the total leucocyte count averaged 7500 per mm^3 and the ratio of neutrophiles to lymphocytes averaged 56% to 45%, respectively. After feeding active supplements to the deficient animals the total leucocyte count averaged 8800 per mm^3 and the neutrophiles were reduced to an average of 30% with a proportionate increase in the number of lymphocytes to 67%.

Figures 1 and 2 illustrate typical curves for the body weight changes of animals developing a deficiency and showing subsequent response to therapy. Depigmented matted underfur was observed in all foxes receiving only the basal ration.

[2] Smaco vitamin test.

TABLE 1

The effect of feeding various supplements to foxes deficient

TABLE 1

The effect of feeding various supplements to foxes developing a deficiency of the unidentified factor(s).

FOX NO.	RATION	TOTAL DAYS ON EXPERIMENT PRIOR TO SUPPLEMENT	SUPPLEMENT	DAYS AFTER SUPPLEMENT	BODY WT. CHANGE AFTER SUPPLEMENTATION	HEMOGLOBIN		NEUTROPHILES/LYMPHOCYTES	
						Before	After	Before	After
					kg	*gm %*	*gm %*	*%*	*%*
13 ♀	Basal	210	10% fresh raw liver	28	+ 0.6	13.3	18.8	37/61	35/65
22 ♂	Basal	336	1% methanol extract [1]	35	+ 0.9	13.3	16.8	70/30	33/67
10 ♂ [2]	Basal	136	10 ml Sharpe and Dohme liver extract [3]	14	— 0.1	12.8	12.6	68/31	51/46
10 ♂	Basal + double vitamin level	157	10% fresh raw liver	56	+ 2.7	12.6	16.9	51/46	45/55
9 ♂ [2]	Basal	131	30 ml Sharpe and Dohme liver extract [3]	56	— 1.9	17.3	16.5	46/54	70/29
9 ♂	Basal	187	1% methanol extract [1]	56	+ 1.3	16.5	17.7	70/29	27/70
18 ♀	Basal	175	6% dried brewers' yeast	70	+ 2.0	13.2	13.5	78/21	21/79
18 ♀	Basal + 6% yeast	245	50 ml Sharpe and Dohme liver extract [3]	53	0	13.5	13.9	21/79	21/78
18 ♀	Basal + 6% yeast	298	1% methanol extract [1]	26	0	13.9	16.5	21/78	14/86
11 ♀	Basal	224	6% dried brewers' yeast	35	0	14.3	14.5	14/86	31/67
11 ♀	Basal + 6% yeast	259	1% methanol extract [1]	28	0	14.5	16.1	31/87	43/57
40 ♂ [2, 4]	Basal	357	6% residue [5]	50	— 0.5	14.9	16.8	58/40	51/46
40 ♂	Basal + 6% residue	427	4% residue [5] + 2% methanol extract [6]	56	+ 0.8	14.5	17.7	20/80	42/56
35 ♂ [2, 4]	Basal + double the vitamin level	285	10% fresh raw liver	56	+ 2.7	15.2	18.8	43/54	21/77
21 ♀	Basal + double the vitamin level + ascorbic acid 50 mg/day	98	10% fresh raw liver	63	+ 2.7	11.8	16.2	33/67	16/82
26 ♀	Basal + 3% whole liver substance [6]	112	6% whole liver substance [6]	49	+ 1.3	14.7	16.8	19/81	31/62
33 ♂ [4]	Basal + 2% "1:20" L.E.P. [7]	364	3% "1:20" L.E.P. [7] (new sample)	42	+ 1.3	16.2	17.0	42/55	40/60
34 ♂ [4]	Basal + 2% "1:20" L.E.P. [7]	344	3% "1:20" L.E.P. [7] (new sample)	21	— 0.2	13.1	12.3	46/48	45/48
34 ♂		365	Raw whole milk, 50 ml/day	35	+ 1.7	12.3	16.7	45/48	28/70
2 ♂	Basal + 3% whole liver substance [6]	154	10% fresh raw liver (removed whole liver substance)	84	+ 2.5	11.4	16.5	61/30	42/55
6 ♂	Basal + 6% whole liver substance [6] + ascorbic acid 50 mg/day	140	Raw whole milk, 50 ml/day	70	+ 1.8	10.4	15.8	66/32	40/58
14 ♀	High protein (30% casein)	210	1% methanol extract [1]	28	+ 0.5	14.5	17.0	53/41	34/66

[1] Methanol soluble fraction of fresh liver, 1 ml equivalent to 10 gm fresh liver.

[2] Animals recovered from previous folic acid deficiencies.

[3] Sharpe and Dohme — refined liver extract no. 2505, 1 ml per day injected intramuscularly.

[4] Adult animals.

[5] Residue of fresh liver (methanol insoluble fraction).

[6] Wilson's whole liver substance.

[7] Wilson's "1:20" liver extract powder.

Those fed supplements of fresh liver or dried liver powders had well-developed prime pelts. Autopsy of the 7 animals which died of the deficiency (table 2) revealed yellow, enlarged, fatty livers and degenerate pale-colored kidneys.

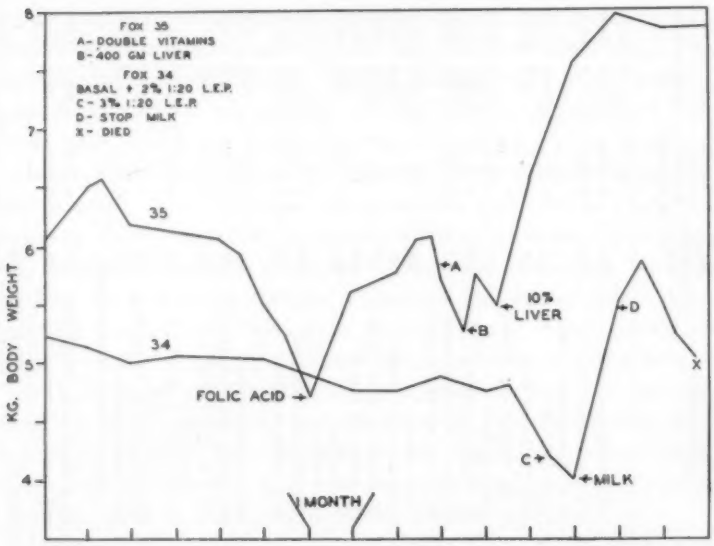

Fig. 1 Body weight curves of adult foxes 34 and 35. Fox 35 was started on the basal ration (without folic acid); folic acid therapy was started at 0.5 mg per day and continued to point A; A = fed the basal ration plus double the vitamin supplement until point B; B = started the basal ration and 400 gm of fresh beef liver fed over a period of 1 week. Fox 34 was started on the basal ration minus folic acid plus 2% "1: 20" liver extract powder; folic acid included in the ration after the animal was on experiment 5 months; when the milk supplement (50 ml/day) was started "1: 20" liver extract powder was discontinued.

To eliminate the possibility that fresh liver or milk merely supplied additional amounts of the known crystalline vitamins, or of protein, the following supplementations were made: Foxes 10, 21 and 35 received daily twice the amount of the vitamin supplement per 100 gm of ration. Fox 8 was fed 30% casein instead of 19%, in addition to the double level of vita-

mins. Foxes 6, 7 and 21 received 50 mg ascorbic acid per 100 gm ration. In all animals these supplements neither alleviated nor prevented the deficiency syndrome.

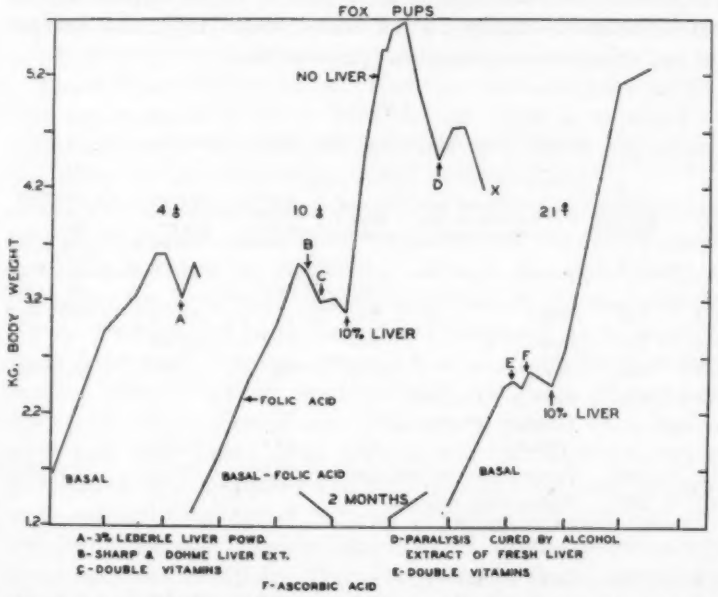

Fig. 2 Body weight curves of fox pups 4, 10 and 21. Fox 4 died after a slight response to Lederle liver powder. Fox 10: B = 10 ml of Sharpe and Dohme liver extract no. 2505 injected intramuscularly; D = paralysis cured by the oral administration of 15 ml of methanol extract and 4 ml intraperitoneally over a period of 1 week; therapy was discontinued as the animal recovered from the paralysis; X = died. Fox 21: E = started feeding twice the vitamin supplement given in table 1; this was discontinued when 10% fresh liver was added to the ration; F = started 50 mg ascorbic acid per 100 gm of ration.

The results with foxes 2, 6, 26, 33 and 34 indicate that the whole liver substance [3] fed at 3 to 6%, and "1:20" liver extract powder fed at 2 to 3% did not contain the activity supplied by 10% fresh liver and that the potency varies in different samples of the same product.

[3] Wilson's.

Since most of the deficient animals had a suboptimal hemoglobin concentration, a refined liver extract of the antipernicious anemia factor was administered to foxes 9, 10 and 18. The results indicate that at the levels of 10, 30 and 50 ml, injected intramuscularly (2 ml every other day), the extract did not stimulate hemoglobin regeneration.

TABLE 2

Animals that died of the liver factor deficiency.

FOX NO.	TOTAL DAYS ON EXP'T.	RATION AND SUPPLEMENT PRIOR TO DEATH	LOSS OF BODY WEIGHT	HEMOGLOBIN AT TIME OF DEATH	NEUTROPHILES/ LYMPHOCYTES	LIVER ANALYSIS	
						fat wet basis	H_2O
			kg	*gm %*	%	%	%
3 ♂	145	Basal	0.2	7.2	49/51	34.5	54.9
10 ♂	287	Basal [1]	0.5	15.7	55/42	...	...
34 ♂ [2]	390	Basal [3]	0.9	15.7	27/65	13.3	67.3
38 ♂ [2]	294	Basal + 0.4 mg folic acid per 100 gm ration	0.8	16.6	75/25	...	...
7 ♀	121	Basal + ascorbic acid	1.0	16.3	45/36	19.2	60.3
8 ♀	252	High protein ration + double vitamin level	0.3	17.0	71/28	...	...
4 ♂	142	Basal + 3% liver preparation [4]	0.2	9.2	72/27	11.7	72.9

[1] After 1 response to fresh liver, the supplement was withdrawn from the ration.
[2] Adult foxes.
[3] After 1 response to raw whole milk the supplement was withdrawn from the ration.
[4] Lederle.

In a search for other sources of the liver factors, the ration for foxes 18 and 11 was supplemented with 6% dried brewers' yeast. At the time of therapy, fox 18 had lost 1 kg of body weight, hemoglobin was 13.2 gm % and the neutrophile-lymphocyte ratio was 78/21. Eight weeks after feeding yeast, the body weight had increased from 3.3 to 5.3 kg and the neutrophile-lymphocyte ratio had returned to a normal of 21/79 for foxes. However, supplementation of the ration with yeast

or yeast plus 50 ml refined liver extract no. 2505[4] (injected intramuscularly) failed to correct the anemia. The ration for fox 11 was supplemented with 6% yeast at the time the hemoglobin value had decreased to a suboptimal level with body weight normal. No response in hemoglobin formation was noted, but normal body weight was maintained.

These observations indicate that 2 or more unidentified factors are supplied by fresh liver, one of which is required for maintenance of body weight (present in yeast or liver) and the other for optimum hemoglobin concentration.

The studies of Jaffe and Elvehjem ('47), on the fractionation of the growth-stimulating factor in liver for rats on a yellow corn-soybean meal ration, offered the possibility of following the activity of similarly prepared concentrates for foxes. Extracts of fresh liver were prepared as follows: 10 kg of fresh beef liver was ground in a meat grinder and thoroughly stirred for 45 minutes with 10 liters of absolute methanol. The mixture was filtered by means of a canvas cloth and filter press. The residue was extracted twice with 10 liters of 60% methanol and filtered. The combined filtrates were concentrated under reduced pressure (35 mm Hg) to a volume of 2 liters. The filtrate was then extracted twice with equal volumes of ether. The ether soluble fraction was discarded and the filtrate concentrated under vacuum to approximately 1 liter; pH was adjusted to 6.8; the solids were removed by filtration and discarded. The volume of the solution was adjusted to 1 liter so that 1 ml of extract was equivalent to 10 gm of fresh liver. Total solids of the extract were 0.31 to 0.35 gm per milliliter. This preparation was fed at a level of 1 ml per 100 gm of ration (which is referred to as 1% methanol extract). The residue of fresh liver, after the methanol extraction, was dried at 34°C. to 40°C. for 30 hours and finely ground.

The activity of the methanol extract was measured on animals 9, 11, 14, 18 and 22. At the time of the deficiency syndrome, when anorexia and paralysis were evident in pups 9,

[4] Sharpe and Dohme.

14 and 22, 3 ml of methanol extract was administered orally and 1% added to the ration thereafter. The hemoglobin level was increased to an optimum level in all the animals. Foxes 9 and 22 gained 1300 and 900 gm, respectively, but failed to attain their previous normal body weight. After this initial response in body weight to 1% methanol extract, the supplement was increased to 2% and finally 3%. The animals again showed a loss of body weight. Further supplementation of the ration for fox 9 with 4% residue plus 1% methanol extract resulted in a gain of 900 gm body weight. Fox 14 was sacrificed for its pelt after the initial response to the methanol extract, thus excluding additional observations as to whether this preparation contained only a portion of the activity of fresh liver. When the feeding of yeast to foxes 18 and 11 did not alleviate the anemia, their rations were supplemented with 1% methanol extract. Within a period of 3 to 4 weeks the hemoglobin level increased from 13.9 to 16.5 gm % in fox 18 and from 14.5 to 16.1 gm % in fox 11.

When the first decline in body weight was noted in fox 40, the ration was supplemented with 6% residue (methanol insoluble fraction of fresh liver), but loss in body weight continued. When anorexia developed the ration supplement was changed to 4% residue plus 2% methanol extract. After 4 weeks on this regimen body weight had increased to the previous normal value and hemoglobin increased from 14.5 to 17.7 gm %.

DISCUSSION

Fresh raw liver and raw whole milk are good sources of the unidentified factors required for the normal nutrition of foxes. Yeast apparently contains 1 factor which is essential for growth and maintenance of body weight, but is lacking or deficient in a factor necessary for the prevention of anemia.

Preliminary evidence indicates that the methanol soluble fraction of fresh liver contains an active factor(s) which is essential for optimum hemoglobin concentration and normal body weight. A comparison of the animals receiving the

methanol extract with foxes 9 and 40, which received the extract plus the methanol insoluble residue indicates that at least 2 factors are present in fresh liver. Since the antipernicious anemia liver extract failed to stimulate hemoglobin formation in the deficient foxes, the present unidentified factor(s) apparently is not related to the dog anemia factor reported by Ruegamer et al. ('47). The similarity between the monkey anti-anemic factor reported by Cooperman et al. ('45, '46), and the unidentified fox factor(s) is indicated by the general blood changes, loss in body weight and response to similar crude materials.

SUMMARY

1. Fox pups and adults require an unidentified factor(s), distinct from the known crystalline vitamins, for normal nutrition.

2. The deficiency syndrome produced by a lack of this factor(s) is characterized by anorexia, loss in body weight, depigmented, matted underfur, suboptimal hemoglobin levels, reversal of the neutrophile-lymphocyte ratio, fatty, light-colored livers and death.

3. Supplementation of the ration with 10% fresh raw liver or 50 ml per day of raw whole milk corrected the deficiency symptoms.

4. Preliminary evidence indicates that there may be more than 1 factor in fresh liver required by the fox.

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THE REQUIREMENT OF UNIDENTIFIED FACTORS FOR MINK[1]

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ONE FIGURE

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INTRODUCTION

In previous studies on the nutritional requirements of mink (Schaefer et al., '46) it was shown that when these animals were maintained on purified rations supplemented with all the known crystalline vitamins (including folic acid), a deficiency syndrome occurred characterized by anorexia, loss in body weight, suboptimal hemoglobin levels, paralysis and improper furring followed by sudden death. The feeding of fresh raw liver or raw whole milk prevented or alleviated the deficiency. The requirement of unidentified factor(s) for mink is similar to that observed in foxes (Schaefer et al., '47). In this paper we wish to present data on the requirement and fractionation of the unidentified factors for adult mink and kits.

EXPERIMENTAL

Thirteen adult mink and 40 kits were used in these studies. The housing, care and handling of the mink have been described (Schaefer et al., '46). The composition of the purified basal ration (diet I) employed is the same as that used in the

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fox studies (Schaefer et al., '47). A number of animals were fed a high protein ration, where casein was increased to 30% at the expense of sucrose (diet II), and 10% gelatin added at the expense of sucrose (diet III).

Studies with adult mink

Adult mink receiving the purified rations developed deficiency symptoms characterized by loss in body weight, depigmented and poor quality fur, suboptimal hemoglobin levels and anorexia, in periods of 132 to 259 days. The data presented in table 1 show the response in body weight gain and increase in hemoglobin concentration upon addition of various supplements.

Hemoglobin determinations were made on 7 deficient animals before supplementation and again 8 weeks after. Six animals showed suboptimal levels ranging from 10.7 to 15.3 gm %, which increased 3.0 to 5.6 gm % upon the feeding of fresh raw liver or raw whole milk. A suboptimal hemoglobin concentration of 15.3 gm % was also observed in mink 7 and 21, whereas animals 9 and 16 had levels of 19.0 and 16.7 gm %, respectively. Total red and white blood cell counts were not altered in a specific trend. It should be emphasized that occasionally animals died very suddenly without the usual forewarning of anorexia, paralysis and lowered hemoglobin. Adult mink receiving only the purified rations died, and autopsy revealed severe fatty degeneration of livers and kidneys.

The results with mink 2, 7, 8 and 11 indicate that liver fraction L,[2] and "1:20" liver extract powder fed at 2%, and whole liver substance fed at 3%, did not contain as much of the unknown factor(s) as is supplied by 10% fresh liver. In these animals the time of onset of the deficiency syndrome was about 38 weeks, in comparison with the average time of 25 weeks for mink receiving only the purified rations without liver supplements. The results with mink 6 and 19 indicate

[2] Wilson's.

TABLE 1

Studies on the unidentified factor required by adult mink.

MINK NO.	RATION	INITIAL WT.	TOTAL DAYS ON EXP'T. PRIOR TO SUPPLEMENT	SUPPLEMENT AT THE TIME OF DEFICIENCY SYNDROME	DATA BEFORE SUPPLEMENT AND 8 WKS. AFTER			
					Body wt.		Hemoglobin	
					Before	After	Before	After
		gm			*gm*	*gm*	*gm %*	*gm %*
9 ♀[1]	Diet I	860	259	None (died)	410	...	19.0	...
21 ♂	Diet I	1020	161	None (died)	800	...	15.3	...
11 ♀	Diet I + 3% W.L.S.[2]	600	260	Milk ad lib. for 7 days	430	565	...	...
			325	6% W.L.S.[2]	520	585	...	...
20 ♂	Diet I + 6% W.L.S.[2]	1230	286	None (no deficiency syndrome)	1250	...	19.0	...
22 ♂	Diet II	1070	161	10% fresh liver	735	1110	14.9	17.9
16 ♀[1]	Diet II	600	168	Milk ad lib.	475	625	16.7	17.4
17 ♂[1]	Diet II	1120	372	None (died)	830	...	...	...
1 ♂[1]	Diet III	1130	132	10% fresh liver	750	1140	10.7	16.3
6 ♀[1]	Diet III	660	216	10% fresh alfalfa	645	570	17.3	13.7
			272	20% fresh liver	570	650	13.7	17.3
19 ♀[1]	Diet III	640	154	Fresh tomato extract[3]	540	500	15.5	15.3
			210	10% fresh liver	500	660	15.3	19.4
2 ♂	Diet III + 2% "1:20" L.E.P.[4]	990	259	Milk ad lib.	740	1120	13.5	17.5
7 ♀	Diet III + 2% fraction "L"[5]	650	315	None (died)	550	...	15.3	...
8 ♂	Diet III + 2% fraction "L"[5]	1110	231	10% fresh liver	900	1250	14.5	17.9

[1] Animals which had a previous folic acid deficiency.

[2] Wilson's whole liver substance.

[3] Filtrate of fresh ground tomatoes fed at a level equivalent to 50 gm fresh tomatoes per day.

[4] Wilson's "1:20" liver extract powder.

[5] Wilson's fraction "L" liver extract.

that fresh tomatoes and alfalfa, at the levels fed, were inactive in alleviating the deficiency. Mink 17, receiving diet II, did not exhibit a severe loss of body weight; however, erratic body weight changes and depigmented matted underfur were noted after approximately 100 days on experiment. The animal died suddenly after being on experiment for 372 days.

Studies with mink kits

The mink kits were weaned at 8 to 9 weeks of age and fed the basal ration supplemented with 20 gm of stock ranch meat ration for 3 days. After this period the animals were weighed and placed on the purified diets. Weight of the kits at the start of the experiment varied from 325 to 415 gm for females and from 395 to 650 gm for males.

The survival time of all mink fed diets I or II without further supplementation varied from 34 to 325 days. Average data for the deficient animals and for the controls receiving supplements of fresh liver or milk are shown in table 2.

TABLE 2

Mink kits receiving purified rations with and without liver supplements.

	RATION	TOTAL DAYS ON EXP'T.	BODY WEIGHT	HB	LIVER ANALYSIS	
					fat wet basis	H_2O
			gm	*gm %*	%	%
Mink kits which died of the deficiency						
Average of 4 (♂)	Diet I	260	807	17.4	48.6	38.0
Average of 3 (♀)	Diet I	54	405	14.8[1]	...	...
Mink no. 65 (♂)	Diet II	210	755	...	18.5	76.0
Mink kits — controls receiving liver or milk supplement						
Average of 5 (♂)	Diet I + 20% fresh liver	260	1210	19.8	8.6[2]	61.3[2]
Average of 3 (♀)	Diet I + 20% fresh liver	56	700	18.1	5.8	66.8
Mink no. 69 (♀)	Diet I + 25 ml raw whole milk per day	56	650	17.7	...	...

[1] Hemoglobin determined on 1 ♀.

[2] Liver fat and moisture analysis on 1 male.

Severe, fatty, yellow-colored, enlarged livers and pale-colored kidneys were observed in kits which died of the deficiency. Kits receiving the purified rations developed a depigmented, matted underfur. Once this improper furring developed it was not possible to produce a black, densely furred pelt by feeding sources of the liver factor(s); however, improvement in the general texture of the fur was noted. That this lack of pigmentation was not a genetic mutation was established by maintaining these animals on the purified ration supplemented with liver throughout the shedding cycle. The new underfur and guard hair were of good texture and black in color.

The responses in body weight and hemoglobin concentration of some of the mink when various supplements were fed are presented in table 3. The results with mink 31 receiving 10% fresh liver indicated that the requirement of the liver factor(s) was greater for kits than for adults. Gain in body weight was very rapid for 56 days, but this was followed by general failure and loss of body weight. When the level of fresh liver was increased to 20% of the ration, a sustained increase in body weight resulted until maturity was reached. A similar response was obtained with female no. 64.

Whole liver substance [3] fed at 3% and 6% gave results similar to those with adult mink. Fresh beef pancreas failed to produce a response in mink 62, but in another male (mink 58) a slight increase of 85 gm body weight occurred in 28 days. The activity of fresh raw milk is illustrated in the case of mink 45, which received the supplement of 25 ml per day added to the water. The same response was observed with mink 69. Both of these animals showed good growth, a good fur coat and a normal blood picture. Upon autopsy, however, degeneration in both the liver and kidney was noted. The activity of fresh raw whey is indicated in the case of mink 48. Dried brewers' yeast fed at a 6% level to kits 33 and 38 resulted in a gain in body weight; but body weight was not maintained. The same methanol extracts of fresh liver that were found to be active for foxes (Schaefer et al., '47) were

[3] Wilson's.

TABLE 3

Response to supplements administered to mink kits at the time of deficiency symptoms.

MINK NO.	RATION	SUPPLEMENT ASSAYED	TIME INTERVAL		BODY WT.		HEMOGLOBIN	
			Before supplement or change in supplement	After supplement	Before	After	Before	After
			Total days on exp't.	*Days interval*	*gm*	*gm*	*gm %*	*gm %*
55 ♀	Diet I	20% fresh liver	56	42	375	500	14.8	19.2
31 ♂	Diet I + 10% fresh liver	20% fresh liver	63	98	775	1320	15.5	19.6
24 ♂	Diet I	6% W.L.S.[1]	102	14	820	1000	18.8	...
		20% fresh liver	168	35	965	1250	...	19.3
62 ♂	Diet I	20% fresh beef pancreas	94	11	660	500	16.7	15.8
		20% fresh liver	105	133	500	1200	15.8	18.9
45 ♀	Diet I	Raw whole milk	63	70	400	800	14.6	18.4
48 ♂	Diet I	Raw whole whey [2]	119	84	665	945	...	...
38 ♀	Diet I	6% dried brewers' yeast	126	21	550	670	....	17.6
		6% yeast + 2% methanol extract [3]	161	28	585	705	17.6	20.0
39 ♂ [4]	Diet I	4% residue [5]	220	42	725	885	19.7	...
		8% residue	278	7	775	700	...	...
		4% residue + 1% heated methanol extract [6]	285	28	700	835	...	...
67 ♂	Diet I	Double amount of vitamin supplement	210	112	855	700	16.9	16.7
		1% heated methanol extract	322	14 [7]	700	775	16.7	17.0

[1] Wilson's whole liver substance.

[2] Raw whole whey fed at equivalent of 50 ml whole milk.

[3] Methanol soluble fraction of fresh liver, 1 ml equivalent to 10 gm fresh liver.

[4] Animal having a previous folic acid deficiency.

[5] Residue of fresh raw liver (methanol insoluble fraction).

[6] 50 ml methanol extract autoclaved at 15 lbs. pressure for 15 minutes, neutralized and filtered. Total solids = 0.15 gm per ml.

[7] Animal accidentally killed.

used in these studies. After mink 33 showed anorexia for a period of 3 days, 2 ml of methanol extract of fresh liver was fed orally and 1% (1 ml equivalent to 10 gm fresh liver per 100 gm of ration) mixed into the ration thereafter. Three weeks later body weight had increased from 564 to 725 gm, and increasing the methanol extract to 2% resulted in an additional 75 gm weight gain. Mink 38, after responding to

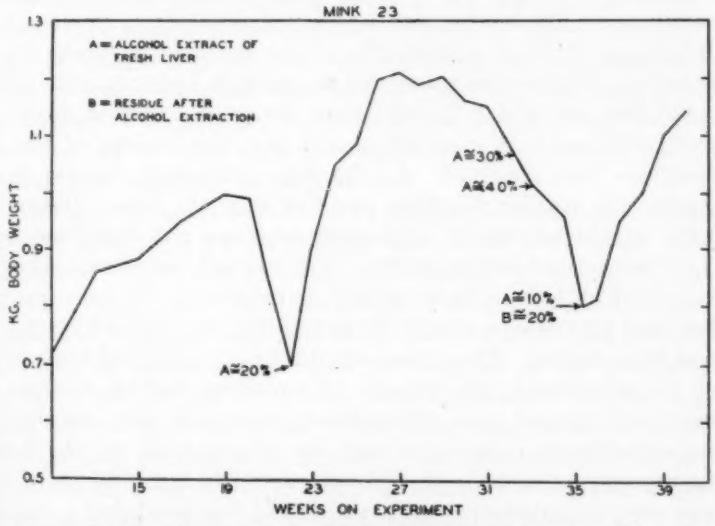

Fig. 1 Body weight curve of mink 23, showing the response in body weight gain, first to the methanol soluble fraction, and then to the methanol insoluble fraction of fresh raw liver.

6% dried brewers' yeast, again lost body weight and the additional symptom of paralysis of the rear quarters was noted. The oral administration of 2 ml methanol extract for the first 3 days of therapy, and supplementing the ration with 2% thereafter, cured the paralysis and the animal gained 120 gm weight.

Preliminary evidence indicating the existence of 2 separate active fractions in fresh raw liver was obtained with animals 23, 39 and 44. Figure 1 shows the body weight curve of mink

[1] Animal having a previous ruffle acid deficiency.

23. The administration of 2% methanol extract of fresh liver (equivalent to 20% fresh liver) produced a dramatic increase of 500 gm body weight within 4 weeks. When loss in body weight again became evident the level of methanol extract fed was increased to 3%, and finally 4%. Loss in body weight continued and after anorexia had occurred for 3 days, 2 gm of the residue of liver (methanol insoluble material) was fed orally in a watery paste for 4 days. It was then mixed into the ration at an 8% level, which was approximately equivalent to 20% fresh liver, along with 1% of the methanol extract. The resulting response as shown by the gain in body weight indicated that at least 2 factors were involved. Similar results were obtained with mink 39, except that the feeding of the 2 fractions was reversed. As the first deficiency became apparent the residue fraction was fed at a 4% level. Gain in body weight was again temporary and was not sustained by increasing the residue to 8%. The second deficiency, characterized by loss in body weight, paralysis of the rear quarters and anorexia, occurred 65 days after the residue therapy had been started. The ration was then supplemented with 1% of an autoclaved (15 pounds of pressure for 15 minutes) methanol extract plus 4% residue. Anorexia and paralysis were alleviated and gain in body weight resulted. As the first sustained loss in body weight was noted in mink 44 the diet was supplemented with 8% residue, and this produced a slight gain of 90 gm weight. Fifty-five days after this continued therapy the animal died suddenly. Mink 67 (table 3) receiving the basal ration plus twice the vitamin supplement developed anorexia, paralysis and loss of body weight. The ration was then supplemented with 1% heated methanol extract. Anorexia and paralysis were alleviated and body weight increased. Two weeks after initiation of therapy the animal was accidentally killed.

DISCUSSION

It is evident from these data that mink are unable to survive upon a purified diet containing sucrose, casein, mineral salts,

cottonseed oil, cod liver oil and all the known crystalline vitamins. The maintenance or gain of body weight in adult and young growing mink is only temporary and once the stores in the body are depleted, nutritive failure and death result. Suboptimal hemoglobin levels (mainly noted in adults), the grayish-matted, dull-appearing fur, and paralysis of the hind quarters, are outward indications of upset metabolism. The length of time elapsing between the start of the experiment and a severe loss in body weight or death varied in adults from 132 to 259 days and for kits from 34 to 325 days. The average survival time for 24 mink kits was 140 days. It should be pointed out again that each mink should be considered individually with a recognition of the genetic factors which may influence the experiment.

During the course of these experiments it became evident that if mink kept on the purified diets were to be saved from certain death, it was necessary to administer fresh raw liver or raw whole milk. Further studies indicated that 6% dried brewers' yeast contains part of the activity furnished by fresh liver. The animals responded to yeast, as noted by a gain in body weight, but when they were maintained on this regimen failure again occurred. The observation reported in the fox studies (Schaefer et al., '47) that the methanol soluble fraction of fresh raw liver was active in correcting this second deficiency was tried and found successful in mink.

Preliminary observations indicate that several factors required by mink are present in fresh raw liver. Initial separation of the activity in fresh raw liver by methanol extraction indicates that the methanol soluble fraction contains 1 factor or factors and that the methanol insoluble fraction contains another factor or factors.

SUMMARY

1. Mink kits and adults fed highly purified rations supplemented with all the known crystalline vitamins require additional unidentified factors for normal nutrition. The characteristic deficiency symptoms are described.

2. Preliminary evidence from the fractionation of the unidentified factors in fresh raw liver indicates that methanol extraction removes 1 factor(s) and that the insoluble residue contains another factor(s).

ACKNOWLEDGMENTS

We wish to acknowledge our indebtedness to Merck and Company, Rahway, New Jersey, for the crystalline vitamins; to Lederle Laboratories, Inc., Pearl River, New York, for the synthetic folic acid; and to Wilson Laboratories, Chicago, Illinois, for the various liver preparations.

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SUPPLEMENTAL VALUE OF CERTAIN AMINO ACIDS FOR BEEF PROTEIN

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Beef is an important source of animal protein in the American dietary. In 1940 it supplied 41% of the protein in all kinds of meat, including meat by-products, produced in the United States (Rose et al., '42). In 1944–45, meat, including poultry and fish, supplied 28% of the protein contributed by the major food groups consumed in this country (Clark et al., '47).

The protein in beef is generally considered to be highly digestible and of relatively high biological value, but there is some evidence indicating that beef may be deficient in certain amino acids. For example, Mitchell and Smuts ('32) conducted paired feeding tests with young rats to determine the effects of the addition of 0.25% cystine to diets containing 8 and 20%, respectively, of beef protein. The addition of cystine to the diet containing 8% protein induced significantly better growth than the control diet, but the addition of cystine to the diet containing 20% protein was without material effect.

In a recent paper Mitchell and Block ('46) present a comparison of the amino acid content of various proteins, with egg as a standard. In this paper (p. 601), the data indicate that beef muscle is apparently deficient in cystine and in the following essential amino acids: phenylalanine, tryptophane, methionine, leucine, isoleucine, valine, and possibly threonine. The maximum deficiency is for cystine, followed by cystine +

methionine. Cow's milk shows similar deficiencies in cystine and methionine. These deficiencies are based on chemical or microbiological analyses and some of the apparent deficiencies remain to be tested by animal experimentation.

The biological value of egg protein is not stated in the paper by Mitchell and Block ('46); however, in an earlier paper by Mitchell and Carman ('26) a biological value of 94 was reported when egg protein was fed to young rats at the 8% level. Sumner ('38) found a biological value of 97 for egg when tested with young rats fed a diet in which protein constituted 8%.

Mitchell and Block ('46) cite a biological value of 76 for beef muscle when protein constituted 7.6% of the diet. Hoagland and Snider ('46) reported an average growth-promoting value of 3.15 gm gain per gram of beef protein in 30-day tests with young rats as compared with a gain of 4.28 gm by rats fed dried egg when the diets contained 10% protein.

In view of the apparent deficiency of beef protein in a number of essential amino acids, as compared with egg protein, it seemed desirable to test these apparent deficiencies by appropriate feeding trials with young rats. The purposes of these experiments were to determine (1) whether beef protein was deficient in phenylalanine, tryptophane, methionine, leucine, isoleucine, valine, or cystine, and (2) the effects of the addition of adequate quantities of these amino acids on the growth-promoting properties of the proteins. For comparative purposes, egg protein was tested under similar conditions.

EXPERIMENTAL

Products tested

Two lots of dehydrated beef were prepared at different times. Lot 1 was prepared from the rounds (rump and shank off) of a steer having a live weight of 910 pounds and a chilled carcass weight of 537 pounds. The carcass graded "low Good." Lot 2 was prepared from the rounds of a steer having a live weight of 872 pounds and a chilled carcass weight of

525 pounds, with the carcass grade of "high Commercial." The rounds were boned out and the lean meat was trimmed free of extraneous fat and connective tissue. The meat was cut into small pieces, frozen at 0°F., ground, and dehydrated at 120°F. to a moisture content of less than 10%. The dehydrated beef was thoroughly extracted with ethyl ether at room temperature. The extracted meat was ground fine and stored in tightly covered tin cans at 25°F. or lower.

Two lots of dried eggs were prepared as follows: fresh eggs were boiled 10 minutes, chilled in cold water, shelled, ground through a meat grinder, and dehydrated at a maximum temperature of 140°F. The dried eggs were thoroughly extracted with ethyl ether, ground, and stored under the same conditions as the dehydrated beef. The dehydrated eggs and beef were analyzed for moisture, ash, fat and nitrogen.

Diets fed

In experiment 1, table 1, the diets were prepared as follows: Dehydrated beef and eggs were incorporated in the diets in such proportions as to supply 10, 12.5, or 15% protein, and certain amino acids were added as indicated. The following quantities of B vitamins were added to 100 gm of diet: thiamine hydrochloride, 0.3 mg; riboflavin, 0.3 mg; pyridoxine hydrochloride, 0.6 mg; calcium pantothenate, 1.5 mg; and choline chloride, 40 mg.

The fat-soluble vitamins were added contained in lard in such amounts that 1 gm of the diet would contain 5 I.U. of vitamin A, 1 unit of vitamin D, and 20 μg of alpha-tocopherol. Sufficient kettle-rendered lard was added to make 10% fat in the diet. Salt mixture amounting to 4% and sufficient dextrin to make 100% completed the diet.

The following amino acids were added to the diets: L-cystine, and the DL forms of each of the following: methionine, phenylalanine, leucine, isoleucine, valine, and tryptophane. In the case of leucine, isoleucine, and valine, only one-half of the nitrogen content of each amino acid was charged against the

nitrogen content of the diet, since only the natural isomers of these amino acids are effective with the rat.

In experiment 2, table 2, cystine was added to diets containing 10% beef protein in the proportions of 0.2, 0.3, 0.4, and 0.5%. For comparative purposes, diets were prepared to contain the same percentages of total nitrogen from beef alone as were present in the corresponding diets containing added cystine.

The feeding tests with rats were conducted in the manner previously described by the writers ('47).

RESULTS

Experiment 1

In table 1 are summarized the results of feeding tests with young rats to determine the supplemental value of 6 essential amino acids, and of cystine, for the protein in beef when protein constituted 10% of the diet. The supplemental value of cystine for beef protein was tested also at 12.5 and 15% levels of intake. Egg protein was fed at the 10, 12.5, and 15% levels for comparative purposes.

A statistical analysis of the experimental data was made by means of Fisher's "t" values for the significance of differences between the means of the gain in weight per gram of nitrogen consumed. The following probability values were obtained: diet 1 with diets 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16 and 18 — very highly significant ($< .001$); diet 12 with diet 17 — very highly significant ($< .001$); diets 1 and 12 with diet 13 — highly significant ($< .01$); diet 2 with diet 3 — not significant; diet 4 with diet 5 — not significant; diet 3 with diet 16 — not significant; diet 13 with diet 17 — not significant; diet 14 with diet 18 — not significant.

The results of the tests with diets 1 to 5, inclusive, indicate very clearly that beef protein was deficient in either cystine or methionine when protein constituted 10% of the diets. When either amino acid was added to a diet containing 10% beef protein, there was a marked increase in actual gain in

TABLE 1

Supplemental value of certain amino acids for the protein in dried raw beef in 30-day tests with male albino rats.

DIET NO.[1]	DESCRIPTION OF DIETS				Nitrogen in diets	GAIN IN WEIGHT	FEED CONSUMED	FEED CONSUMED FOR 1 GM GAIN IN WEIGHT	GAIN PER GM OF NITROGEN CONSUMED
	Protein		Amino acids added to diets						
	Kind	*%*	*Kind*	*%*	*%*	*gm*	*gm*	*gm*	*gm*
1	Beef [2]	10.0	.....	...	1.60	118	337	2.86	21.9
2	Beef	10.0	Cystine	0.20	1.62	146	368	2.52	24.6
3	Beef	10.0	Cystine	0.40	1.65	154	370	2.40	25.3
4	Beef	10.0	Methionine	0.20	1.62	145	362	2.50	24.8
5	Beef	10.0	Methionine	0.40	1.64	149	358	2.40	25.3
6	Beef	10.0	Methionine Phenylalanine	0.40 0.20	1.65	148	358	2.42	25.1
7	Beef	10.0	Methionine Leucine	0.40 0.40	1.66	152	365	2.40	25.1
8	Beef	10.0	Methionine Isoleucine	0.40 0.40	1.66	142	342	2.41	25.0
9	Beef	10.0	Methionine Valine	0.40 0.40	1.66	143	349	2.44	24.6
10	Beef	10.0	Methionine Tryptophane	0.40 0.10	1.65	138	347	2.51	24.1
11	Beef	10.0	Methionine Phenylalanine Leucine Isoleucine Valine Tryptophane	0.40 0.20 0.40 0.40 0.40 0.10	1.74	140	338	2.41	23.9
12	Beef	12.5	.....	...	2.00	155	393	2.54	19.6
13	Beef	12.5	Cystine	0.40	2.05	163	380	2.33	20.9
14	Beef	15.0	.....	...	2.40	165	384	2.33	17.9
15	Beef	15.0	Cystine	0.40	2.45	160	379	2.37	17.2
16	Egg	10.0	.....	...	1.60	149	373	2.50	24.9
17	Egg	12.5	.....	...	2.00	158	366	2.32	21.6
18	Egg	15.0	.....	...	2.40	164	374	2.28	18.2

[1] Each diet, except no. 2, was fed to 8 rats. Diet no. 2 was fed to 7 rats.

[2] Lot 1 of dehydrated beef was used in the experiments reported in table 1.

weight by the rats, and in gain in weight per gram of nitrogen consumed (growth-promoting value). Cystine was just as effective as methionine in supplementing the protein in beef. Although the results in table 1 appear to indicate that 0.4% of either cystine or methionine was slightly more effective than 0.2%, the differences in growth-promoting values were not significant.

The results of the experiments with diets 6 to 10, inclusive, indicate that beef protein was not deficient in either phenylalanine, leucine, isoleucine, valine, or tryptophane when the diets were also supplemented with methionine. The results with diet 11 appear to indicate a depression in growth-promoting value when all the above-named amino acids were added, but this result is due to the increase in nitrogen content of the diet from the addition of 5 amino acids that were not utilized for growth. If the growth-promoting value of diet 11 is calculated on the basis of the nitrogen content of a diet containing 10% protein and 0.4% methionine (diet 5), then the values for the 2 diets are practically the same. The feed consumed per gram gain in weight is practically the same for diets 5 and 11.

When beef protein was increased to 12.5% (diet 12), there was a marked increase in the rate of growth compared to that with the diet containing 10% protein, but the gain in weight per gram of nitrogen consumed was significantly lower. Diet 12 was still somewhat deficient in cystine, since the addition of this amino acid induced a significant increase in growth-promoting value.

When beef protein was increased to 15% (diet 14), there was a further increase in the rate of growth, but a decrease in growth-promoting value. However, the feed consumed per gram gain in weight for this diet was very low as compared with that for most other diets. The addition of cystine to diet 15 did not increase its growth-promoting value. Apparently, 15% beef protein supplied ample cystine and methionine for optimum growth in rats.

The experiments with eggs show that the protein was utilized most efficiently when the diet contained 10% protein, but the actual gain in weight was largest when the protein level in the diet was 15%.

A comparison of the growth-promoting value of the protein in beef with that in egg shows that egg protein was definitely superior to beef protein when the diets contained either 10% or 12.5% of protein, but no supplementary amino acids. However, when beef protein was supplemented with either cystine or methionine, the growth-promoting values of beef protein and egg protein were practically the same. When the diets contained 15% protein but no added cystine, beef protein and egg protein had approximately the same growth-promoting value.

Experiment 2

In table 2 are summarized the results of experiments to determine the supplemental effects of different proportions of

TABLE 2

Supplemental value of different proportions of cystine for the protein in dried raw beef in 30-day tests with male albino rats.

DIET NO.[1]	DESCRIPTION OF DIETS					GAIN IN WEIGHT	FEED CONSUMED	FEED CONSUMED FOR 1 GM GAIN IN WEIGHT	GAIN PER GM OF NITROGEN CONSUMED
	Protein		Amino acids added to diets		Nitrogen in diets				
	kind	*%*	*kind*	*%*	*%*	*gm*	*gm*	*gm*	*gm*
19	Beef [2]	10.00	.....	...	1.60	118	349	2.96	21.1
20	Beef	10.00	Cystine	0.20	1.62	139	360	2.59	23.9
21	Beef	10.13	.....	...	1.62	127	366	2.88	21.4
22	Beef	10.00	Cystine	0.30	1.63	151	376	2.49	24.6
23	Beef	10.19	.....	...	1.63	120	349	2.91	21.0
24	Beef	10.00	Cystine	0.40	1.65	146	366	2.51	24.2
25	Beef	10.31	.....	...	1.65	116	338	2.91	20.8
26	Beef	10.00	Cystine	0.50	1.66	144	354	2.46	24.6
27	Beef	10.38	.....	...	1.66	120	344	2.87	21.1

[1] Each diet, except no. 20, was fed to 8 rats. Diet no. 20 was fed to 7 rats.

[2] Lot 2 of dehydrated beef was used in the experiments reported in table 2.

cystine on the growth-promoting value of beef protein when the diets contained 10% protein. Statistical tests of the experimental data were made in the same manner as for experiment 1 with the following probability values: diet 19 with diet 20; diet 20 with diet 21; diet 22 with diet 23; diet 24 with diet 25; and diet 26 with diet 27 — all *very highly* significant ($< .001$); diet 20 with diets 22, 24 and 26 — all *not* significant. The results of experiment 2 confirm those of experiment 1, i.e., that the addition of cystine to a diet containing 10% beef protein greatly improved its growth-promoting value. Apparently the addition of 0.2% cystine was nearly, if not quite, sufficient for optimum results since there was no significant increase in growth-promoting value when larger quantities of cystine were added to the diets.

DISCUSSION

The experimental data presented in this paper indicate that cystine was as efficient as methionine in supplementing the protein in beef so as to increase its growth-promoting value to that of egg. These results raise the question as to the relative importance of the 2 amino acids in the diet of the young rat. On the basis of the values for cystine and methionine in beef protein and in egg protein given by Mitchell and Block ('46), a diet containing 10% of either protein would contain the following proportions of the 2 amino acids:

Egg: methionine, 0.41%; cystine, 0.24%; cystine and methionine, 0.65%. Ratio methionine to cystine, 1.7 : 1. Cystine as per cent of total cystine and methionine, 36%.

Beef: methionine, 0.33%; cystine, 0.13%; cystine and methionine, 0.46%. Ratio methionine to cystine, 2.5 : 1. Cystine as per cent of total methionine and cystine, 28.3.

Beef + 0.2% cystine: methionine, 0.33%; cystine, 0.33%; cystine and methionine, 0.66%. Ratio methionine to cystine, 1 : 1. Cystine as per cent of total methionine and cystine, 50.

The above data indicate that egg protein, which has a very high biological value at the 8% level, contains methionine and

cystine in the proportion of 1.7 : 1, whereas beef protein with a considerably lower biological value contains these amino acids in the ratio of 2.5 : 1. However, when beef protein was supplemented with 0.2% cystine, the ratio of methionine to cystine was 1 : 1, and the growth-promoting value of the mixture was equal to that of egg protein. The total quantity of cystine and methionine in the egg diet was practically the same as that in the beef + cystine diet. These results indicate that the young rat can utilize methionine and cystine very efficiently for maintenance and growth even when the ratio between the 2 amino acids is 1 : 1. These findings are in contrast to those of Womack and Rose ('41) who conducted experiments with young rats on diets containing pure amino acids as the source of nitrogen. They reported that only a small portion (approximately one-sixth) of the methionine requirement could be satisfied by cystine. The ratio of methionine to cystine under those conditions would be 5 : 1.

Attention is called to the fact that in the experiments herein reported beef protein was found to be deficient only in cystine or methionine, whereas Mitchell and Block ('46) indicate that beef protein is also deficient in phenylalanine, tryptophane, leucine, isoleucine, valine, and possibly threonine. It is possible that beef protein might have been found deficient in amino acids other than cystine or methionine if protein had been fed at a lower level of intake than 10%. Whatever the explanation may be, the results of our experiments with beef protein indicate the importance of checking apparent amino acid deficiencies in proteins, as determined by analysis, by experiments with suitable animals. It is highly important also to determine the level of protein intake at which an amino acid deficiency ceases to exist.

SUMMARY

The supplemental values of the amino acids cystine, methionine, phenylalanine, leucine, isoleucine, valine, and tryptophane for beef protein were determined by feeding tests with young male albino rats.

When protein constituted 10% of the diet, beef protein was deficient only in cystine or methionine. When beef protein was supplemented with 0.2% to 0.4% of either cystine or methionine, the growth-promoting value of the protein was equal to that of egg protein.

When the diet contained 12.5% protein, beef protein was somewhat deficient in cystine, but when this amino acid was added, the growth-promoting value was increased to that of egg protein.

When protein constituted 15% of the diet, beef protein was not deficient in cystine and the growth-promoting value was equal to that of egg protein.

The ratio of methionine to cystine in a diet containing 10% egg protein was 1.7 : 1; in a diet containing 10% beef protein the ratio was 2.5 : 1; and in a diet containing 10% beef protein + 0.2% cystine the ratio was 1 : 1.

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THE ISOLEUCINE REQUIREMENT OF THE INFANT[1]

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ONE FIGURE

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A survey of the literature indicates that on a body weight basis the nitrogen needs of the infant are 4 to 5 times higher than those of the adult (Albanese, '47). However, the available data do not reveal whether the higher protein requirements of the infant arise from a proportionate increase in all of the amino acids or from a limiting effect created by greater demands of the growing organism for 1 or several of the amino acids. The lack of and the need for this information, which is of obvious importance to practical infant dietetics and the physiology of growth in general, led us to undertake investigations on the amino acid needs of the infant. The determination of the isoleucine requirement of the infant is reported here.

METHODS AND DIETS

In this as in previous studies (Albanese et al., '47), the requirement of an essential amino acid was estimated from the additional amount needed to restore to physiological levels the nitrogen retention and rate of body weight gain of subjects

[1] The work described in this report was supported by grants from the Rockefeller Foundation, National Live Stock and Meat Board, and Mead Johnson and Company.

previously maintained on a deficient diet. On the basis of preliminary experiments the following regimen was used: For a week preceding the deficient period the subjects were maintained on a complete diet as constituted by an evaporated milk formula in the first experiment and the deficient diet reinforced with isoleucine in the second experiment. After the deficient diet had been fed for 3 weeks, isoleucine was added in a stepwise manner to a maximum of 8% of the protein intake. Seven-day intervals elapsed between each isoleucine increment tested.

The observations presented here were made on 5 normal healthy male infants who were given the synthetic diets in 5 feedings daily at the rate of approximately 100 cal. per kilogram of body weight and 500 mg of ascorbic acid together with 15 drops of oleum percomorphum daily. The diet periods were of 7 days' duration and consecutive, but collections were omitted on week-ends to avoid complications which might arise from the continued use of restraints. The subjects were immobilized by the use of abdominal restraints which were also designed to hold the urinary adapters in place. Twenty-four-hour urine specimens were collected in bottles containing 1 ml of 15% (by volume) HCl and 1 ml of 10% alcoholic thymol. The feces were collected in 19 cm porcelain evaporating dishes which were held in place by a properly shaped excavation in the mattress, and the daily stools were accumulated under refrigeration for each period in jars containing 200 ml of 70% alcohol. The subjects were weighed daily during the course of the experiment.

The composition of the diets employed is shown in table 1. The diets contained approximately 100 cal. per 100 gm, the percentage caloric distribution being as follows: protein, 14; fat, 36; carbohydrate, 50. The protein moiety of the isoleucine deficient diet was prepared by sulfuric acid hydrolysis of dried beef hemoglobin.[2] This digest which was processed by the procedure previously employed by us in the preparation of human hemoglobin hydrolysates (Albanese and Barnes, '45)

[2] Armour.

was found by analysis to contain 0.60% of isoleucine. The protein equivalent of the final product was estimated as N × 6.25. The product was then supplemented with 1.5% of L-tryptophane to replace that which was destroyed during acid hydrolysis. The cystine-poor characteristic of the preparation was improved by the addition of 1% of L-cystine.

TABLE 1

Composition of diets.

(All diets fed at the rate of 100 cal. and 3.5 gm of protein per kilogram body weight.)

DIETS	A	B	C	D	E	F
	gm	gm	gm	gm	gm	gm
Acid hydrolyzed beef hemoglobin [1]	3.40	3.30	3.30	3.30	3.20	3.10
L-Tryptophane	0.06	0.06	0.06	0.06	0.06	0.06
L-Cystine	0.04	0.04	0.04	0.04	0.04	0.04
L-Isoleucine	0.00	0.02	0.05	0.09	0.18	0.27
Brewers' yeast [2]	1.00	1.00	1.00	1.00	1.00	1.00
Olive oil	4.00	4.00	4.00	4.00	4.00	4.00
Dextri-maltose #2	9.60	9.60	9.60	9.60	9.60	9.60
Arrowroot starch	2.30	2.30	2.30	2.30	2.30	2.30
Salt mixture [3]	1.60	1.60	1.60	1.60	1.60	1.60
Water	78.00	78.00	78.00	78.00	78.00	78.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Estimated isoleucine content (mg)	36	60	90	126	216	306

[1] N × 6.25 = gm of protein containing 0.60% of isoleucine by chemical analysis.

[2] Kindly supplied by the Mead Johnson and Company and found to contain 3.0% isoleucine by chemical analysis.

[3] The salt mixture employed had the following composition (measured in gm): $FeSO_4$ 0.9, NaCl 6, Ca gluconate 48, $Ca(OH)_2$ 12, KH_2PO_4 7, KCl 6, and MgO 0.1.

The isoleucine supplement was added to the diet in the stepwise fashion (table 1). Owing to uncertainties regarding the complete human requirements of B complex vitamins, brewers' yeast was employed instead of a mixture of the synthetically available vitamins. The quantities of isoleucine derived from this source appear to be approximately 16 mg per gram

(Carter and Phillips, '44). Thus, the amount of isoleucine per kg of infant body weight provided by the diets can be estimated roughly (table 1). The final nitrogen content of each batch of diet was determined by micro-Kjeldahl analysis.

The evaporated milk formula employed for the precontrol period in the first experiment had the following composition: evaporated milk, 40 ml (55.3 cal.); corn syrup, 6 ml (17.7 cal.); water, 54 ml. This supplied approximately 73 cal. per 100 ml and the nitrogen content of each batch was controlled by micro-Kjeldahl analysis.

The data on nitrogen retention were calculated from the results of nitrogen determinations of the 24-hour urine collections, analyses of the pooled feces for each period, and from computation of the daily nitrogen intake based on food consumption records and the known nitrogen content of the diets.

Blood samples (10 ml) were collected over lithium oxalate by vena puncture on the last day of each diet period. The hemoglobin concentration of these specimens was determined colorimetrically in the Klett-Summerson photoelectric colorimeter. The total plasma proteins, albumin, globulin and non-protein-nitrogen were determined by the procedure recently described by us (Albanese, Irby and Saur, '46).

RESULTS AND DISCUSSION

The data obtained from the first experiment of this study (table 2) show that the omission of isoleucine from the diet caused an immediate and sustained drop of the daily weight gain and nitrogen retention below the control levels of all the subjects. Since the growing organism is normally in a state of high positive N balance, a drop from the nitrogen retention values characteristic of the individual must be given the same interpretation as the establishment of a negative N balance in the adult, namely, that isoleucine is a dietary essential for the infant. This inference is also corroborated by the observed decrements in rate of weight gain. Following the deficient period, all 3 subjects were given diet D which provided ap-

proximately one-third of the isoleucine calculated for the evaporated milk formula (Williamson, '44). It will be noted that this diet restored the N retention and weight coefficients to the levels attained with the evaporated milk diet. Since the feeding of diets E and F failed to augment the magnitude of these criteria, it could be concluded that the 126 mg of L-isoleucine per kg of body weight supplied by diet D are adequate (if not more than adequate) for infants of this age.

TABLE 2

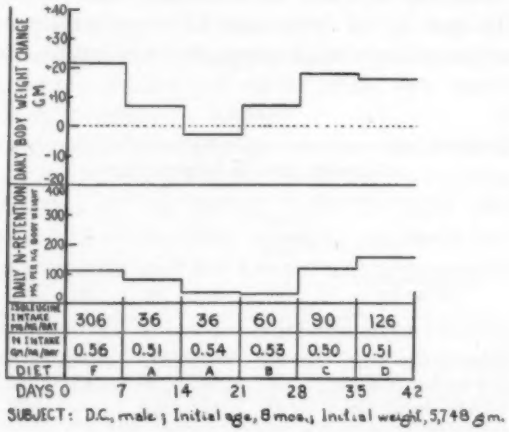
Effect of dietary isoleucine on the nitrogen retention and body weight of the infant.
(All results given as daily averages.)

INITIAL AGE AND WEIGHT OF SUBJECT	DIET	EVAPORATED MILK	A	A	A	D	E	F
	Isoleucine intake, mg/kg/day	306	36	36	36	126	216	306
H. G.,								
male,	N intake (gm)	2.85	2.69	2.90	2.80	2.77	3.10	3.05
4 mos.	N retention (mg/kg)	220	81	65	43	246	248	224
4.735 kg	Weight change (gm)	+31	—21	+7	—4	+28	+36	+23
W. T.,								
male,	N intake (gm)	4.10	4.01	3.83	4.04	4.29	4.13	4.23
10 mos.	N retention (mg/kg)	180	63	92	64	192	164	174
6.965 kg	Weight change (gm)	+30	+21	+4	+9	+47	+42	+30
A. H.,								
male,	N intake (gm)	3.30	3.21	2.99	3.20	3.08	3.26	...
9 mos.	N retention (mg/kg)	200	92	71	59	291	212	...
6.173 kg	Weight change (gm)	+32	—28	0	—28	+28	+28	...

Moreover, the fact that diets D, E, and F sustained N retention levels and weight gains comparable to those found with the evaporated milk formula further suggests that the nitrogen moiety of these diets, namely, the beef hemoglobin hydrolysate as modified for this study, has a biological value similar to that of the casein and lactalbumin mixture found in evaporated milk.

In order to ascertain the isoleucine requirement of the infant more closely than was permitted by our first experiment,

2 more subjects were similarly studied with the exception that diets B and C, which supplied, respectively, 60 and 90 mg of isoleucine per kg, were fed after 2 weeks of the deficient regimen. The results of this experiment (fig. 1) indicate that the N-retention and daily body weight change were restored



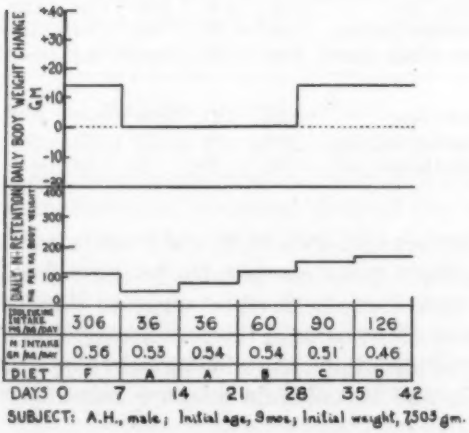


Fig. 1 Effect of variations of isoleucine content of the diet on the nitrogen retention and body weight changes of the infant.

to the control levels by diet C but not by diet B and signify therefore that infants of this age group require approximately 90 mg of L-isoleucine per kilogram per day. This value is approximately one-third of that which is normally supplied by milk formulae fed at comparable nitrogen and caloric levels.

Measurements of the blood proteins during the experiments disclosed that, unlike the tryptophane deficient diet, the isoleucine deficient diet had no appreciable effect on the blood plasma protein and hemoglobin levels of the infant. This finding is of interest in view of the low isoleucine content of these tissue proteins.

Careful clinical examinations of the infants throughout the course of these experiments disclosed that the feeding of an isoleucine poor diet for periods of 2–3 weeks does not induce any detectable clinical symptoms in the infant.

SUMMARY

On the basis of changes in rate of growth and nitrogen retention induced by the fractionally supplemented control diets following 3 weeks of the isoleucine deficient regimen, it has been estimated that the infant requires approximately 90 mg of L-isoleucine per kilogram per day.

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THE ROLE OF VITAMIN D IN THE UTILIZATION OF PHYTIN PHOSPHORUS[1]

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INTRODUCTION

Earlier studies (Spitzer and Phillips, '45a, '45b) demonstrated that the rat could efficiently utilize the phytin phosphorus of soybean oil meal when adequate vitamin D and a favorable Ca-P ratio were employed. These studies indicated that the phytin phosphorus was made available by the action of an intestinal enzyme, phytase, capable of splitting the phytin molecule. In later studies, Boutwell et al. ('46) also suggested the importance of an intestinal phytase in the utilization of phytin phosphorus. These same investigators also showed that the availability of phytin phosphorus was greatly enhanced when vitamin D was added to the ration. Other evidence in the literature has shown the importance of vitamin D in the utilization of phytin phosphorus. Krieger and co-workers ('40, '41) demonstrated that in the absence of vitamin D, phytic acid phosphorus was not available to the rat, but when vitamin D was fed, more of the phosphorus was utilized. In studies with chicks, McGinnis and co-workers ('44) showed that phytin phosphorus was poorly available in

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the absence of vitamin D, but that it was nearly as available as inorganic phosphorus when adequate vitamin D was fed. Singsen and Mitchell ('45) also reported that vitamin D increased the utilization of phytin phosphorus in chicks.

Thus it seems apparent that the availability of phytin phosphorus is dependent upon the action of an intestinal phytase and upon an adequate level of vitamin D in the ration. It occurred to us that vitamin D might be necessary either for phytase formation or for its proper functioning. It seemed possible that if vitamin D was necessary for phytase formation, tissues from rats receiving varying amounts of vitamin D would vary in their phytase content. The following experiments were designed to obtain information about this possible relationship. Phytase activity was measured in rats fed calcium phytate and inorganic phosphorus and varying amounts of vitamin D.

EXPERIMENTAL

Weanling male rats of Sprague-Dawley breeding were divided in 4 lots and fed rations outlined in table 1. The basal ration (lot I) contained 0.26% inorganic phosphorus and was devoid of vitamin D. Lot II received the same ration but in addition was given 100 A.O.A.C. units of calciferol by dropper 3 times each week. Lots III and IV differed from the first 2 lots in that the phosphorus was furnished as calcium phytate.[3] Both of these rations contained 0.23% calcium phytate phosphorus and 0.02% inorganic phosphorus. Lot IV received 100 A.O.A.C. units of calciferol by dropper 3 times weekly.

The phosphorus level in all rations was kept as near as possible to 0.25%, a level slightly below the optimum phosphorus intake for weanling rats (Spitzer and Phillips, '45b). It was felt that this level would afford a more critical measure of the availability of calcium phytate and the effect

[3] Phytin is the calcium-magnesium salt of phytic acid. Phytic acid is the hexaphosphoric acid ester of inositol. These compounds and other salts of phytic acid are probably rendered available by similar mechanisms.

of vitamin D on this process. The calcium content of all rations was kept as near as possible to 0.6%.

Rats were decapitated on the thirty-second day of the experiment. Approximately a 1-gm sample of small intestine was removed, weighed and frozen. In all cases the sample was taken by severing the small intestine at the pylorus and using the duodenum for analysis. The femora were also

TABLE 1

Composition of rations (per 100 gm ration).

BASAL MIXTURE	LOT I	LOT II	LOT III	LOT IV	ALL LOTS: (Vitamins/100 gm basal mixture	
	gm	gm	gm	gm		mg
Blood fibrin	14.0	14.0	14.0	14.0	B-carotene	2.0
Sucrose	77.75	77.75	78.15	78.15	Riboflavin	0.3
Corn oil	4.0	4.0	4.0	4.0	Thiamine HCl	0.3
Low Ca-P salts [1]	2.0	2.0	2.0	2.0	Pyridoxine	0.3
$CaHPO_4$	1.37	1.37	...	...	Ca pantothenate	2.5
$CaCO_3$	0.48	0.48	...	...	Niacin	2.5
Ca phytate	...	...	1.45	1.45	Vitamin D [2]	
Inositol	0.1	0.1	0.1	0.1		
Choline chloride	0.3	0.3	0.3	0.3		
Phosphorus [3] *in %:*						
Inorganic P	0.26	0.26	0.02	0.02		
Phytin P (Ca phytase)	...	...	0.23	0.23		
Total P	0.26	0.26	0.25	0.25		

[1] Low Ca-P salt mixture used earlier by Spitzer and Phillips ('45a).

[2] Rats in lots II and IV were given 100 A.O.A.C. units of calciferol by dropper 3 times each week.

[3] Inorganic P analyses were made, using the method of Fiske and Subbarow ('25). Phytin P analyses were made, using the method of McCance and Widdowsen ('35).

removed and bone ash .values determined as previously described (Spitzer and Phillips, '45a). All rats were starved for 36 hours prior to the time they were sacrificed in order to clear the intestine of food materials. Samples of the small intestine were later removed from the refrigerator and phytase determinations made.

RESULTS

Growth and bone ash studies

Growth and bone ash data are summarized in table 2. Rats receiving the inorganic phosphorus (lots I and II) made more rapid gains than did those receiving the calcium phytate phosphorus (lots III and IV). Rats given vitamin D (lots II and IV) made somewhat poorer gains than did those on the basal ration (lots I and III).

Under the conditions of this experiment, vitamin D supplementation had little or no effect on bone ash values when inorganic phosphorus was fed. This contrasts sharply with the effect on the utilization of calcium phytate phosphorus.

TABLE 2

Effect of vitamin D on growth and bone ash in rats fed inorganic and calcium phytate phosphorus.

LOT NUMBER	NO. OF RATS	PHOSPHORUS Form and amount in ration	AVERAGE BODY WEIGHT AT 32 DAYS FROM INITIAL WEIGHT OF 40	BONE ASH
			gm	%
I	8	Inorganic (0.26%)	122	47.1
II	8	Inorganic (0.26%) + vitamin D	114	48.0
III	8	Calcium phytate (0.25%)	80	23.3
IV	8	Calcium phytate (0.25%) + vitamin D	69	43.4

Supplementation of vitamin D when calcium phytate was fed had a marked effect on the utilization of this organically bound form of phosphorus (lots III and IV). The bone ash values of the rats in lot III averaged 23.3%. These same animals showed marked signs of rickets throughout the experiment. Supplementation with vitamin D (lot IV) markedly increased the utilization of the calcium phytate phosphorus. The bones of these rats contained 43.4% ash and no signs of rickets were observed throughout the study. Considering the slightly lower phosphorus level in the calcium phytate ration (lot IV) as compared to that for the inorganic control (lot II), it appears that the calcium phytate phos-

phorus was nearly as efficiently utilized as was the inorganic phosphorus when adequate vitamin D was fed.

During the course of the experiment a very marked alopecia developed in lot IV. No marked signs of alopecia were observed in the other lots. Lot III rats were especially well-coated.

Development of a phytase method

The phytase method outlined by Spitzer and Phillips ('45b) was adapted for quantitative determination. Frozen tissue of known weight, usually 1 gm, was homogenized by the Potter-Elvehjem technique ('36). The sample was made to volume (25 ml) with distilled water. A suitable buffer and the substrate (sodium phytate) contained in test tubes were diluted to a 9 ml volume and brought to the incubation temperature. To one tube was added 1 ml of the tissue homogenate and to the control tube, 1 ml of distilled water. Using this procedure it was possible to check specifically any phytase activity by measuring the increase in inorganic phosphorus resulting from hydrolysis of the sodium phytate. The medium was mixed thoroughly and incubated at 37°C. Samples were removed at intervals, added to an equal volume of 10.0% trichloroacetic acid and then analyzed for liberated phosphorus according to the method of Fiske and Subbarow ('25). The assays were carried out in dimmed and artificial light in an effort to minimize the possibility of contamination by vitamin D. The increase in inorganic phosphorus is a measure of the phytase activity.

Effect of buffers. A 0.05 M diethylamine citrate buffer inhibited phytase activity. A similar concentration of sodium borate proved to be a satisfactory buffer, but the one which allowed greatest phytase activity was a 0.05 M sodium barbitol. Maximum activity was observed at a slightly alkaline pH. Experiments reported herein were conducted at pH 8 using a 0.05 M sodium barbitol buffer.

Effect of time. Table 3 shows the effect of time on phytase activity. Inorganic phosphorus was liberated very rapidly

during the first few minutes of assay. A slower, steadier rate of liberation was then noted.

Phytase determinations on tissue samples. Results showing the effect of vitamin D on phytase activity in rats fed the experimental rations are summarized in table 3. Phytase analyses were made on samples from 8 rats in each lot. Each listed value represents an average of from 2 to 12 assays.

Tissues from rats fed the different sources of phosphorus and various amounts of vitamin D showed no marked variations in phytase activity. When a 5-minute assay period was

TABLE 3

The effect of vitamin D on phytase activity in rats fed inorganic and calcium phytate phosphorus.

LOT NUMBER	PHOSPHORUS Form in ration	MICROGRAMS INORGANIC PHOSPHORUS LIBERATED PER GRAM OF TISSUE HOMOGENATE [1]						
		1 min.	3 min.	5 min.	15 min.	30 min.	60 min.	120 min
I	Inorganic	....	....	2,500	3,000	....	5,900	8,400
II	Inorganic + vitamin D	....	....	2,300	3,100	....	5,500	7,600
III	Calcium phytate	1,900	2,000	2,500	3,200	3,800	5,700	9,700
IV	Calcium phytate + vitamin D	1,500	1,900	2,400	3,300	4,300	5,900	7,700

[1] Each value listed is an average of from 2 to 12 determinations. Eight animals were used in each lot. No determinations were made at 1 min., 5 min., or 30 min. for lots I and II.

used, an average of 2,300 to 2,500 μg of inorganic phosphorus per gram of tissue was liberated by all samples. At 60 minutes, approximately equal amounts (5,500–5,900 μg per gram of tissue) of phosphorus had been freed by all samples. Small variance was observed between samples from different lots when other assay times were used. However, since there was also some variance in samples from the same lot, no definite difference could be noted. In general, as is indicated in table 3, all samples showed approximately the same phytase activity.

Vitamin D in vitro. Results of a limited number of assays indicated that the addition of vitamin D to tissue homogenates in vitro did not affect phytase activity.

DISCUSSION

The importance of vitamin D in the utilization of organically bound phosphorus is demonstrated. Although calcium phytate phosphorus was poorly available in the absence of vitamin D, it was readily available when adequate D was supplied. These results confirm the findings of earlier investigations by Spitzer and Phillips ('45b), Boutwell et al. ('45), Krieger and coworkers ('40, '41), and others.

In the light of these results, the apparent contradictions of earlier studies (Boutwell et al., '46; Spitzer and Phillips, '45a, '45b) on the availability of phytin phosphorus might be explained on the basis of the vitamin D content of the rations used.

The present studies indicate that although vitamin D is necessary for the utilization of calcium phytate phosphorus, this vitamin is not necessary for the formation of the enzyme phytase which makes possible the splitting of the phytin molecule in the intestine. The small intestines of rats fed inorganic or bound phosphorus with or without vitamin D, all showed equal phytase activity as measured by the phytase assay outlined above. These results indicate that vitamin D apparently does not function by aiding phytase formation.

It is interesting to note that although vitamin D increased the utilization of the calcium phytate phosphorus (table 2), growth was somewhat poorer when this vitamin was fed (lot IV). Rats receiving phytate and vitamin D (lot IV) also showed a marked hair loss which did not occur when only calcium phytate was fed (lot III). The pattern of the hair loss was similar to that of an inositol deficiency described by Cunha et al. ('43) and Spitzer and Phillips ('46). In these studies in which inositol deficiencies were reported, the rations contained considerable phytin. If in the presence of adequate vitamin D phytin phosphorus was readily utilized, and if a

molecule of phytin was completely hydrolyzed, the final products would be 1 inositol and 6 phosphoric acid molecules. Theoretically, this inositol should be available to the rat. Yet, an inositol deficiency developed in our experimental animals even though the ration contained 0.1% inositol in addition to that resulting from the hydrolysis of the calcium phytate. It is possible that the slightly imperfect utilization resulted in an antagonistic intermediate which increased the requirement of inositol.

SUMMARY

The effect of vitamin D on the utilization of calcium phytate phosphorus and on inorganic phosphorus has been studied in the rat.

The addition of vitamin D to a ration containing nearly optimum inorganic phosphorus had little or no effect on phosphorus utilization as indicated by bone ash studies. However, the addition of vitamin D to a ration containing calcium phytate greatly enhanced phosphorus utilization. Therefore, in the presence of adequate vitamin D, bone ash values indicated that the calcium phytate phosphorus was almost as effectively utilized as the inorganic phosphorus.

The phytase content of the small intestines of rats fed calcium phytate and varying amounts of vitamin D showed no difference in phytase activity. Apparently vitamin D was not necessary for phytase formation. It is suggested that vitamin D functions in a secondary role in the utilization of phytin phosphorus.

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THE UTILIZATION BY THE CHICK OF PHOSPHORUS FROM DIFFERENT SOURCES[1]

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Recent changes in animal feeding practices have increased the need for inorganic phosphorus supplements to supply part of the phosphorus formerly obtained from animal protein supplements and steamed bone meal. This has led to a greater production and use of thermally defluorinated superphosphate and phosphate rock. The desirability of defluorinated phosphate products as phosphorus supplements is indicated by the results of a number of workers (Fraser, Hoppe, Sullivan and Smith, '43; McConnell, Insko and Buckner, '44; Barrentine, Maynard and Loosli, '44, and others). Considerable variability has been found to exist, however, among products produced under different conditions. This is particularly true of the superphosphates (Bird et al., '45).

Differences in availability are attributable in large part to variation in the content of pyrophosphate and metaphosphate. In a series of collaborative studies, Ellis et al. ('45) and Bird et al. ('45) have shown that the calcium pyrophosphates and calcium metaphosphates which are likely to occur in defluorinated phosphate products are less available than the ortho form of phosphate. These 3 forms of phosphorus are capable of existing singly or together in the end product following

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the defluorinating process. Furthermore, each of these calcium phosphates, ortho, pyro, and meta, is capable of existing in a number of different enantiotropic crystalline forms and, in the case of the calcium metaphosphate, a vitreous form is also possible. The temperature at which the fluorine is eliminated is a primary factor in determining the type of phosphorus compound formed; however, other factors such as rate of cooling may influence the final crystalline modification or result in a vitreous system.

More exact information would be valuable on the relative biological availability of each of these compounds which may be expected to occur in defluorinated phosphates. This is true not only from the standpoint of determining their individual feeding values, but also in evaluating the over-all availability of the defluorinated phosphate material in which several forms may occur, depending upon the treatment given. At the same time more information is needed concerning the relative value of the commonly-used phosphorus supplements. Accordingly, a series of experiments has been conducted to determine the relative availability of a number of pure phosphate compounds and other common phosphorus supplements when used as sources of phosphorus in a low phosphorus basal diet. The importance of crystalline structure and solubility in determining availability has been investigated also. The results of these studies are reported in this paper.

EXPERIMENTAL DIET AND MATERIALS

Although studies have been made of the relative efficiency of different phosphorus supplements for the chick, most of these experiments are open to possible criticism because the basal diets used have been too high in phosphorus to permit the most efficient study of single supplements. This has been true particularly in the case of studies involving the pyrophosphates and metaphosphates. In many instances, the test supplements have raised the phosphorus level of the diet considerably above the requirement of the chick, whereas it would seem desirable to make comparative studies near the critical

level of phosphorus required. With these facts in mind, a basal diet has been developed which does not contribute a significant percentage of the phosphorus requirement of the chick. In formulating a suitable basal diet low in this element, the principal difficulties encountered are the organic phosphorus in the cereal grains and the phosphorus associated with

TABLE 1

Low phosphorus basal diet.

	%		%
Corn starch	58.5	Vitamin mixture (see below)	0.5
Dried blood fibrin	25.0		
Gelatin	5.0	Salt mixture (see below)	6.0
Ground cellophane	3.0		
Soybean oil	2.0		

SALT MIXTURE	%	VITAMINS	*gm/100 lbs.*
NaCl	0.6	Thiamine	0.15
KCl	0.5 [1]	Riboflavin	0.225
$MgSO_4 \cdot 7\ H_2O$	0.4	Ca pantothenate	0.50
$FeSO_4 \cdot 7\ H_2O$	0.06	Pyridoxine	0.225
$MnSO_4 \cdot 4\ H_2O$	0.03	p-aminobenzoic acid	5.00
$ZnCl_2$	0.001	Niacinamide	0.80
$CoCl_2 \cdot 6\ H_2O$	0.0003	Inositol	50.00
KI	0.0033	Choline	100.00
$CuSO_4 \cdot 5\ H_2O$	0.0017	Tocopherols in oil	70.00
Ca + P supplements [2]		Vitamin K	0.10
		Folic acid	0.15
		Biotin	10 mg
		Vitamin A alcohol	50 mg
		Vitamin D_3	22,700 AOAC chick units

[1] When KH_2PO_4 was used as a source of phosphorus, no KCl was added.

[2] Variable, depending on the supplements used. When necessary, adjustments were made in level of corn starch.

most of the common protein supplements used in chick diets. Following the suggestion of Jones ('38), dried blood fibrin was found to be low in phosphorus as well as an excellent source of protein. The cereal grains were eliminated by using corn starch. The composition of the basal diet used in all the studies reported in this paper is given in table 1. It contains

approximately 0.03% phosphorus or less than one-tenth the amount required by the chick.

The supplements used in these experiments together with their analytical and solubility data are listed in table 2. They include the commonly-used supplements — steamed bone meal, defluorinated superphosphate (defluorination temperature

TABLE 2

Analytical and solubility data for phosphorus supplements.

MATERIAL	Ca	P	% TOTAL P SOLUBLE IN 0.4% HCl [1]
	%	%	
KH_2PO_4	...	22.7	100.0
Beta-tricalcium phosphate	38.5	19.7	84.6
Tricalcium phosphate [2]	36.9	19.0	90.5
Dicalcium phosphate [2]	28.9	22.7	100.0
Monocalcium phosphate [2]	15.3	24.8	100.0
Steamed bone meal	31.8	14.2	97.6
Defluorinated superphosphate	36.1	15.5	86.8
Calcined rock phosphate	32.4	18.6	87.5
Fused rock phosphate	29.3	12.0	89.0
Vitreous calcium metaphosphate	17.6	25.7	54.2
Vitreous sodium metaphosphate	...	29.5	100.0
Potassium metaphosphate	...	26.6	93.5
Beta-calcium metaphosphate	21.3	30.2	3.2
Gamma-calcium metaphosphate	21.3	29.8	5.7
Calcium acid pyrophosphate, crystalline	19.0	28.5	98.6
Alpha-calcium pyrophosphate	31.0	24.0	69.8
Beta-calcium pyrophosphate	31.3	24.1	54.2
Gamma-calcium pyrophosphate	31.3	24.2	73.4
Calcium phytate	12.9	12.6	13.7

[1] Method of Reynolds et al. ('44): 1 gm material digested in 100 ml 0.4% HCl for 1 hour.

[2] Reagent grade material.

1150°C.), calcined rock phosphate, fused rock phosphate, and reagent grades of orthophosphates, mono-, di-, and tricalcium phosphate. Calcined rock phosphate is defluorinated at temperatures below that required for complete melting, and fused rock phosphate freed from its fluorine at temperatures above that needed for complete melting. In addition, a number of pure pyrophosphates, metaphosphates, and pure *beta* tri-

calcium phosphate were included. A crude calcium phytate was used since an important part of the phosphorus in many rations consists of phytic acid and its salts.

The sample of *alpha* calcium pyrophosphate was produced by a commercial source for use in these experiments. All of the other pure pyrophosphates and metaphosphates as well as *beta* tricalcium phosphate were obtained from the U.S.D.A. Agricultural Research Administration, Beltsville, Maryland. A detailed description of these products, together with methods of preparation, has been given by Hill et al. ('44, '45 and '48). It is sufficient to point out that the different Greek letter-designated modifications of calcium metaphosphate [$Ca(PO_3)_2$] and calcium pyrophosphate [$Ca_2P_2O_7$] differ among themselves only in crystal structure and not in chemical composition. The designations begin with the *alpha* form which is stable at the melting point. The *gamma* calcium metaphosphate was only about 80% pure, the impurity being mainly $Ca_2HP_3O_{10}$. The *beta* tricalcium phosphate is that form of pure $Ca_3(PO_4)_2$ which is stable at ordinary temperatures and which predominates in defluorinated superphosphate and calcined rock phosphate. The reagent grades of mono-, di-, and tricalcium phosphate were obtained from commercial sources, and, as Hodge, LeFevre and Bale ('38) have pointed out, they are seldom pure compounds corresponding to the theoretical composition. The alkaline salts, vitreous sodium metaphosphate and potassium metaphosphate, were studied although they are not expected to occur in thermally defluorinated phosphates.

PROCEDURE

White Leghorn cockerels were used as experimental animals. Lots of 15 1-day-old chicks were placed on the experimental diets in electrically heated brooders with food and water supplied ad libitum. All chicks were weighed individually at weekly intervals during the 4-week experimental period. At the end of the fourth week, 10 representative chicks in each lot were killed and the left tibia removed for bone ash

determination. In lots with 10 chicks or less surviving the 4-week experimental period, all survivors were used for bone ash determinations.

Each phosphorus supplement was added to the basal diet at 2 levels calculated to raise the phosphorus content to 0.4% and 0.8%, respectively. The 0.4% level is near the minimum requirement of the chick for phosphorus. It was thought that by supplying phosphorus at this level, a more critical comparison of relative availability would be possible, particularly with the more available phosphates. By increasing the level of phosphorus to 0.8%, any degree of availability in the more unavailable materials should become apparent. Also, it was thought that if a compound exhibited any toxic effects, results should be poorer at the higher level. Where the Ca : P ratio of the supplement added was less than 2 : 1, this ratio was established by the addition of pure calcium carbonate. In the case of bone meal, fused rock phosphate, and the defluorinated superphosphate used, the ratio was unavoidably greater than 2 : 1, being 2.18 : 1 for bone meal, 2.43 : 1 for fused rock phosphate, and 2.25 : 1 for superphosphate. The results presented here were collected from 4 different experiments. Since all studies could not be conducted simultaneously, lots receiving KH_2PO_4 were used as positive controls in each experiment to insure that the experiments were comparable. This phosphate was selected for its known ready availability since it is completely water soluble, and it gave uniformly good results in preliminary studies.

RESULTS AND DISCUSSION

The results obtained on growth, bone ash, and mortality are presented in table 3. When the basal diet was fed without additional phosphorus, the chicks died within a short period. Since there is no report in the literature describing the effects of severe phosphorus deficiency in young chicks, a brief description is appropriate here. The effects of the deficiency are acute, 100% mortality occurring in a few days. The chicks eat well for 3 or 4 days and make small initial gains in

weight. After this, however, there is a rapid decline in appetite. Coincident with the loss of appetite, a general weakness develops and the chicks are reluctant to stand or use their legs. The first mortality usually occurs by the fifth or sixth day and all the chicks are dead by the tenth to twelfth day. During the last stages of the deficiency, the chicks usually lie on their

TABLE 3

The relative availability of different phosphorus supplements for the chick.

SUPPLEMENT	0.4% PHOSPHORUS			0.8% PHOSPHORUS		
	Wt. 4th wk.	Bone ash	Mortality	Wt. 4th wk.	Bone ash	Mortality
	gm	%	%	gm	%	%
None[1]	...	...	100.0	...	...	...
Potassium phosphate, monobasic	296	46.5	4.4	291	45.6	0.0
Beta-tricalcium phosphate	281	44.2	0.0	299	46.3	0.0
Tricalcium phosphate (reagent)	272	44.8	0.0	307	48.2	0.0
Dicalcium phosphate (reagent)	303	43.9	6.7	300	47.2	0.0
Monocalcium phosphate monohydrate	307	45.9	13.4	282	47.2	0.0
Steamed bone meal	250	43.5	0.0	290	48.5	0.0
Fused rock phosphate	241	40.7	13.4	265	47.1	0.0
Defluorinated superphosphate	211	38.7	6.7	279	48.0	0.0
Calcined rock phosphate	211	40.0	6.7	271	47.4	0.0
Calcium acid pyrophosphate	285	41.6	0.0	273	43.5	6.7
Alpha-calcium pyrophosphate	...	...	100.0	...	...	100.0
Beta-calcium pyrophosphate	...	...	100.0	...	...	100.0
Gamma-calcium pyrophosphate	...	...	100.0	...	...	100.0
Vitreous calcium metaphosphate	186	30.9	66.7	247	38.4	33.3
Vitreous sodium metaphosphate	155	32.2	80.0	128	35.3	73.2
Potassium metaphosphate	...	...	100.0	...	...	100.0
Beta-calcium metaphosphate	...	...	100.0	...	...	100.0
Gamma-calcium metaphosphate	...	...	100.0	...	...	100.0
Calcium phytate	...	...	100.0	...	...	100.0

[1] Phosphorus level of diet 0.03%.

sides. These results show that the young chick requires an immediate source of available phosphorus for the maintenance of vital functions. The results are approximately the same whether or not calcium is added to the basal diet.

It is evident from table 3 that all the pure or relatively pure orthophosphates studied were excellent sources of phos-

phorus for the chick when added to the basal diet as the sole phosphorus supplement. KH_2PO_4, pure *beta* tricalcium phosphate, and the reagent grades of mono-, di-, and tricalcium phosphate all permitted growth of from 270 to 307 gm at 4 weeks, and bone ash values of from 43.9% to 46.5%, with very low mortality at the 0.4% phosphorus level. At the 0.8% level, growth ranged from 282 gm to 307 gm, and bone ash values from 45.8% to 48.2%.

Steamed bone meal was slightly less available than the first 5 salts, but was a very good source of phosphorus. The results given for bone meal are an average of 2 samples which varied considerably. The results of these experiments indicate that in some instances bone meal may be less available than is generally thought. When it supplied 0.8% phosphorus, bone meal was as good as pure *beta* tricalcium phosphate.

The results obtained with fused rock phosphate, defluorinated superphosphate and calcined rock phosphate demonstrated the value of these materials as phosphorus supplements. However, they were less available than potassium phosphate or the various calcium orthophosphate salts. They were also less available than bone meal. These 3 products were of equal value when they were used to raise the phosphorus content of the diet to 0.8%. At this level they were only slightly less satisfactory than steamed bone meal or pure orthophosphate. The calcined rock phosphate and defluorinated superphosphate were predominantly *beta* tricalcium phosphate as shown by x-ray analyses, and the fused rock was mostly *alpha* tricalcium phosphate. Apparently, therefore, the crystalline structure has little to do with availability in the case of these compounds.

Of the 4 pyrophosphates studied, only the crystalline calcium acid pyrophosphate was available. This compound was a good source of phosphorus, particularly at the marginal level. At the higher level, the results were less favorable than would be expected on the basis of results at the critical level, growth being actually less and bone ash values only slightly better. It seems probable either that the op-

timum level of phosphorus from this compound is less than 0.8%, or that for best results it should be supplemented with other sources of phosphorus. The availability of this product probably derives from the fact that is it easily hydrated in the body to monocalcium phosphate as follows: $CaH_2P_2O_7 \xrightarrow{H_2O} Ca(H_2PO_4)_2$. The *apha, beta,* and *gamma* calcium pyrophosphates were completely unavailable or very nearly so. This occurred in spite of the fact that they are appreciably soluble in 0.4% HCl. There was no significant difference in the survival time of chicks receiving these supplements and those receiving the unsupplemented basal diet, even when a sufficient quantity of these compounds was used to raise the phosphorus content of the diet to 0.8%. These compounds did not appear to be toxic, however. It is evident that here again the different crystalline forms did not vary in their availability.

Of the 5 metaphosphates studied, only the vitreous calcium metaphosphate and the vitreous sodium metaphosphate exhibited any availability. The 2 crystalline forms of calcium metaphosphate were identical in their lack of availability. Although the vitreous calcium metaphosphate is appreciably soluble in dilute HCl, and has been reported to be somewhat available (Bird and Caskey, '43; Ellis et al., '45), it was a poor source of phosphorus in these studies. Mortality was excessive in both lots receiving this compound, especially in the lot which received 0.4% phosphorus. Growth at the lower level was very poor and all surviving chicks developed severe rickets. In the lot receiving 0.8% phosphorus, survival, growth, and bone ash values were better, but still markedly inferior, and all chicks exhibited gross rickets. These results indicate that some of the phosphorus in vitreous calcium metaphosphate is utilized by the chick but that its comparative availability is very poor.

The alkali metaphosphates, vitreous $NaPO_3$, and crystalline KPO_3, are soluble compounds which might be expected to be available biologically if the animal organism is able to metabolize metaphosphate. The sodium metaphosphate is com-

pletely water soluble while the potassium metaphosphate is 6.2% water soluble and 93.5% soluble in aqueous HCl. In these studies, however, the $NaPO_3$ was very poorly utilized. The few chicks receiving this compound which survived the 4-week experimental period grew very little and were in a very weakened rachitic condition. Shelling ('32) concluded that sodium metaphosphate is biologically inert in the rat; however, our results show that it is very slightly used by the chick but is considerably less available than vitreous calcium metaphosphate. The other alkali metaphosphate studied, KPO_3, was virtually unavailable. No chicks survived the experimental period; however, survival on the higher level was slightly longer than on the unsupplemented basal diet. *Beta* and *gamma* calcium metaphosphates were completely unavailable.

A crude calcium phytate, purchased from a commercial source, was tested for availability in these studies. It is not necessarily exactly comparable to the product as it occurs in natural feedstuffs. The calcium phytate was almost completely unavailable under the conditions of this experiment in which 50 units of pure vitamin D_3 were supplied per 100 gm of diet. No chicks survived the experimental period, but survival was a little better than on the unsupplemented basal diet. These results confirm those of Lowe, Steenbock and Krieger ('39) who found that the addition of phytin to a low phosphorus basal diet resulted in no increase in calcification, and those of Heuser and coworkers ('43) who concluded that a reasonable amount of non-phytin phosphorus must be included in chick rations.

Reynolds, Hill and Jacob ('44) proposed solubility in 0.4% HCl as a test for availability of different phosphorus supplements in the absence of information from actual feeding experiments. It is interesting to compare the solubility data for the supplements used in these experiments with the biological availability of the same materials. On the basis of the results reported in this paper, it would seem that solubility in 0.4% HCl has a limited usefulness in estimating availability

for animals. It can readily be seen that not a single one of the compounds which are relatively insoluble in this reagent is available for the chick. On the other hand, a comparative solubility cannot be taken as an indication of availability. Thus, the rather soluble *alpha, beta,* and *gamma* calcium pyrophosphates are completely unavailable biologically. The alkali metaphosphates constitute another example of soluble compounds which are not available. It appears, therefore, that solubility tests have only a negative value in that they may be used to eliminate certain insoluble compounds, but cannot be used to predict availability. It is difficult to understand why compounds which are comparatively soluble under conditions found in the digestive tract of animals should exhibit so little availability. It is to be remembered, however, that crystalline phosphates are capable of existing in a variety of polymerized forms (Hendricks, '44). When metaphosphates go into solution, covalent bonds as well as electrovalent forces must be broken. This results in particles of various sizes breaking off in the dissolving medium. It seems probable, therefore, that the type of sol may have much to do with determining the availability of the different phosphates. If the material presented to the absorbing membrane is in the form of hydrated micelles rather than ions in solution absorption would be impossible.

SUMMARY

The comparative availability of a number of common phosphorus supplements and pure phosphate compounds has been studied by adding them singly to a basal chick diet very low in phosphorus. When used to raise the phosphorus content of the basal diet to 0.4%, the orthophosphates, including KH_2PO_4, pure *beta* tricalcium phosphate and reagent grades of mono-, di-, and tricalcium phosphate, were excellent sources of phosphorus and slightly more available than steamed bone meal. At the same phosphorus level, the defluorinated superphosphate and defluorinated phosphate rock products used in

these experiments were good sources of phosphorus, but less available than the pure orthophosphates or steamed bone meal.

When used to raise the phosphorus content of the diet to 0.8%, the defluorinated superphosphate and defluorinated phosphate rock, both fused and calcined, were of equal value and were nearly as effective as pure *beta* tricalcium phosphate or steamed bone meal.

Crystalline calcium acid pyrophosphate was well utilized by the chick, while vitreous calcium metaphosphate was poorly utilized and vitreous sodium metaphosphate was only slightly available. Potassium metaphosphate, *beta* and *gamma* calcium metaphosphate, *alpha, beta,* and *gamma* calcium pyrophosphate, and crude calcium phytate were virtually unavailable. Comparison of the biological availability of the products used in these experiments with their solubility in 0.4% HCl shows that the solubility test is useful only in that insoluble compounds may be eliminated. The results also show that there was no difference in the availability of the different crystalline forms of a given chemical compound.

Severe phosphorus deficiency in the young chick results in early loss of appetite, weakness, and death within a period of 10 to 12 days.

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AVAILABILITY OF RIBOFLAVIN OF ICE CREAM, PEAS, AND ALMONDS JUDGED BY URINARY EXCRETION OF THE VITAMIN BY WOMEN SUBJECTS[1]

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Information that will aid in distinguishing between the total content of specific vitamins in food materials and the relative amounts of those vitamins which can be utilized by the human subject should lead to a better understanding of how to attain adequate nutrition. Studies designed to determine the relative availability of vitamins from food sources have yielded a variety of findings. Fresh yeast (Parsons and associates, '45) contains an appreciable amount of thiamine which cannot be utilized by man. The extent to which biotin may be absorbed from liver and yeast apparently differs (Gardner et al., '46). Ascorbic acid seems readily available in several foods rich in this vitamin, as indicated by studies in which red raspberries, tomatoes, cabbage, apples, or papayas and guavas served as the test foods (Todhunter and Fatzer, '40; Clayton and Borden, '43; Hauck, '45; Anders,

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'46; Hartzler, '45). These observations make it apparent that knowledge of the vitamin content of the diet is only 1 step toward knowing whether the individual consuming it is well-fed.

Melnick and coworkers ('45) have suggested a human bioassay technic for the determination of the physiological availability of several water-soluble vitamins. The method depends upon the establishment of a constant urinary excretion of the particular water-soluble vitamin under study by normal subjects ingesting an adequate diet providing an excess of the vitamin in question. Then, when extra amounts of the pure vitamin are added to the diet, increased quantities are promptly excreted in the urine. The availability of a vitamin present in various food sources can then be measured by comparing the rise in the urinary output of the vitamin when the nutrient is supplied as a pure chemical and as a test food.

The technic proposed by Melnick and associates has been used in this study and results are reported on the relative availability of riboflavin present in 3 foods.

EXPERIMENTAL

Subjects

Eight women considered to be in good health participated in the experiment. Data concerning their age, height, and weight are given in table 1. The subjects continued their usual activities, proceeding with their graduate studies, assisting in the research laboratory, or teaching.

TABLE 1

Age, height and weight of subjects.

SUBJECT	AGE	HEIGHT	WEIGHT	SUBJECT	AGE	HEIGHT	WEIGHT
	yr.	*inches*	*lb.*		*yr.*	*inches*	*lb.*
HW	21	67	125	EW	26	63	110
HB	32	63	123	GB	19	62	115
RM	29	66	134	MC	20	60	125
SK	23	64	126	GE	37	67	135

Experimental plan

Each subject served as her own control during a series of 5 experiments. In period I the magnitude and constancy of the urinary excretion of riboflavin were determined when the subjects ate the same mixed diet for a period of 12 days. In 4 subsequent 3-day periods the relative availability of the crystalline vitamin and that of the riboflavin in vanilla ice cream, frozen green peas and almonds were measured (periods II, III, IV, V).

During each study the subjects quantitatively consumed a weighed diet which provided approximately 2.4 mg of riboflavin daily. In each 3-day test period, the first day served as an adjustment interval and no urine was collected. On the second day, a 24-hour specimen of urine was collected to establish the amount of riboflavin excreted when the basal diet was consumed. On the third day, 1 mg of riboflavin was added to the basal diet either in the form of a solution of pure riboflavin or as that quantity of test food that contained 1 mg of the vitamin. The supplement was divided into 2 equal portions; one was taken at 10 A.M., and the other at 2:30 P.M. A second 24-hour specimen of urine was collected on the third day to determine the quantity of riboflavin excreted as a result of the administration of the supplement. The experimental days started at 7 A.M.

Each metabolism study was preceded by a 7-day preliminary period. During this time the subjects were allowed to select their own diets except that each was asked to consume sufficient protein from specified protein-rich foods to provide at least 75 gm daily. Since earlier studies had indicated that certain subjects were consuming too little protein, this precaution was found necessary to avoid a temporary storage of both nitrogen and riboflavin when the weighed diet was ingested later. The practice of consuming generous amounts of protein during the preliminary period prevented any further evidence of unusual retention of riboflavin.

During the first 6 days of the preliminary period, all subjects ingested a capsule containing 2 mg of riboflavin. No

food was consumed after 8 P.M. of the evening preceding the experimental studies. No medications were allowed during any portion of the investigation. The water intake was kept uniform during all collection periods.

One subject (GE) repeated the procedure outlined for period II for a total of 4 trials to determine the amount of variation which occurred in the urinary excretion of riboflavin when 1 mg of the pure vitamin supplemented the basal diet.

Composition and preparation of the basal diet

The basal diet (table 2) was planned not only to meet the recommended allowances of the Food and Nutrition Board of the National Research Council ('45) but to provide a surplus of riboflavin. Canned products were purchased in sufficient amounts to cover the needs of the entire study.

TABLE 2

Composition of basal diet.

Breakfast			
Orange juice [1]	150 gm	Milk	400 gm
Whole wheat bread	20 gm	Sugar	10 gm
Cornflakes	15 gm	Butter	10 gm
Dinner			
Lima beans [1]	100 gm	Milk	200 gm
Round steak	100 gm	Peaches [1]	150 gm
Whole wheat bread	20 gm	Peanut butter cookies	40 gm
Supper			
Rice (dry wt.)	30 gm	Apple sauce [1]	150 gm
Round steak	100 gm	Whole wheat bread	20 gm
Tomatoes [1]	150 gm	Butter	20 gm
Peanut butter cookies	40 gm	Sugar	15 gm
		Milk	200 gm
Lunch (Before 9 P.M.)			
Peanut butter cookies	40 gm		
Estimated composition:			
Protein	97 gm	Thiamine	1.4 mg
Fat	138 gm	Niacin	24.1 mg
Carbohydrate	282 gm	Ascorbic acid	110.0 mg
Calories	2758	Riboflavin	2.1 mg

[1] Used in the canned form.

Specially ground lean round of beef, which was purchased in 50-lb. quantities was mixed, wrapped as 100 gm patties, frozen and held at —5°F. until needed. Whole wheat bread from 1 baking was purchased in an amount to exceed the needs of 1 experimental study and it was held at —5°F.

A lot of milk adequate for the entire series of studies was frozen in the college market milk laboratories after the usual pasteurization and homogenization. It was likewise held at —5°F. until needed. Butter was supplied from 1 churning. Peanut butter cookies were prepared in the laboratory, standardized procedures of mixing and baking being followed.

In preparing the food to be used for each metabolism study, quantities of canned products sufficient for the experiment were opened, thoroughly mixed, and stored in sterilized jars in a refrigerator. All foods were weighed directly into the dishes in which they were served. Cooked foods were carefully heated so that both the length and degree of heating were kept constant. Precautions were taken at all times to avoid loss of riboflavin due to exposure to sunlight.

The 8 subjects consumed the same total amount of food daily and no refusals were encountered.

Supplements

It was recognized that the response of the subjects might be altered by the fact that the ice cream, frozen green peas, and almonds selected as the first test foods contributed different quantities of carbohydrate, fat, and protein when consumed in amounts to provide 1 mg of riboflavin. Investigation of the influence of adding riboflavin-free sources of carbohydrate or fat to the basal diet indicated that extra amounts of either nutrient produced a change in the urinary excretion of riboflavin (Walker, '47). This was particularly true when fat served as the supplement. Increasing the protein by supplementing the diet with vitamin-free casein did not alter the urinary excretion of riboflavin.

To maintain the same daily total intake of carbohydrate, protein, and fat throughout all phases of each study the basal

diet was modified to include vitamin-free sources of these nutrients. Eighty gm dextrose, 100 gm butter [2] and 50 gm vitamin-free casein were needed to provide an excess of these nutrients above the quantities that would be encountered in supplying 1 mg of riboflavin from any of the 3 test foods. The sucrose, butter, and peanut butter cookies of the original basal diet were omitted to prevent an excessive intake of calories.

Each test food was assayed for riboflavin, total lipids, and nitrogen. Carbohydrate was estimated from published values (U. S. Bur. Human Nutrition, '45). The composition of the test foods is given in table 3.

TABLE 3

Composition of test foods.

FOOD	WEIGHT CONSUMED	RIBOFLAVIN	CARBOHYDRATE	PROTEIN	FAT
	gm	*mg*	%	%	%
Ice cream	377	1	20.9 [1]	4.1	11.2
Green peas	714	1	12.0 [1]	5.1	0.2
Almonds	112	1	6.5 [1]	22.8	54.0

[1] Available carbohydrate.

The supplement of pure riboflavin was consumed as a solution of the vitamin. The ice cream was prepared in the ice cream laboratory of the dairy industry department and all details of its composition were known. Frozen green peas were thawed slightly, then thoroughly mixed, and refrozen. They were heated for 30 minutes in an oven regulated at 425°F. before serving and were considered "done" but not overcooked. Almonds were served whole as the roasted unblanched nut meat.

Collection of urine

The 24-hour samples of urine were collected in amber bottles containing 50 ml of glacial acetic acid. During the

[2] The butter used contained 30 μg riboflavin per 100 gm.

day all specimens were refrigerated at 36°F.; at night they were held at room temperature. The urine was diluted to a volume of 1500 ml. An aliquot of the diluted 24-hour samples was refrigerated until all analyses could be completed, which was within 10 days of collection in all cases.

Analytical methods

An aliquot of the diet was prepared on the first and last days of the 12-day period (period I) and on the second day of each 3-day study. The riboflavin content of the diet as well as that of all test foods was determined by the microbiological method (Snell and Strong, '39; Strong and Carpenter, '42). The riboflavin content of the urine was determined by a modification of the fluorometric method described by Najjar ('41). Corrections for non-riboflavin fluorescent compounds were made by exposing the samples to bright sunlight for at least 10 hours to destroy the riboflavin. Nitrogen was determined by the Kjeldahl-Gunning procedure and the factor 6.25 was used for the conversion of nitrogen to protein. Total lipids were determined by continuous extraction with ether for 72 hours.

EVALUATION OF METHOD AND RESULTS

Daily urinary excretion of riboflavin

The quantity of riboflavin excreted daily when the subjects consumed the basal diet supplying approximately 2.4 mg of riboflavin per day was surprisingly constant for the 12 days of period I. The day-by-day variation encountered was 11% or less in all subjects except HB and GE. The data are given in table 4. In 6 of the 8 subjects the amount of riboflavin excreted ranged from 770 to 1090 μg per day, equivalent to 33 to 43% of the intake. Subject HW showed a characteristically high excretion of the vitamin, 55% of the ingested riboflavin being eliminated in the urine. In contrast, subject GE excreted approximately 25% of the riboflavin of the diet.

The total daily excretion of riboflavin by the majority of the subjects agreed closely with that reported by Brewer et al. ('46) for college women ingesting approximately 2.7 mg of riboflavin daily. The excretion of the vitamin also approached the upper range of values reported by Keys and associates ('44) and by Melnick and coworkers ('45) for men

TABLE 4

Daily urinary excretion of riboflavin when the basal diet was ingested.

SUBJECT	HW	HB	RM	SK	EW	GB	MC	GE
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Riboflavin excreted								
Day 1	1.25	1.06	0.83	0.77	1.07	0.92	1.09	0.68
2	1.25	1.01	0.82	0.82	1.04	0.86	0.99	0.66
3	1.26	1.02	0.82	0.78	1.03	0.82	0.95	0.63
4	1.34	0.99	0.81	0.77	1.03	0.85	0.97	0.66
5	1.38	0.89	0.82	0.79	1.01	0.82	0.98	0.56
6	1.36	0.89	0.82	0.80	1.01	0.81	0.96	0.56
7	1.38	0.92	0.81	0.80	1.01	0.82	0.96	0.55
8	1.35	0.94	0.80	0.78	0.99	0.78	0.90	0.58
9	1.36	0.94	0.81	0.79	1.01	0.82	0.92	0.58
10	1.30	0.97	0.77	0.79	0.97	0.82	0.91	0.55
11	1.28 [1]	0.97 [1]	0.77 [1]	0.79 [1]	1.03 [1]	0.86 [1]	0.90 [1]	0.57
12	1.25	1.01	0.77	0.83	0.98	0.81	0.89	0.58
Per cent variation [2]	10.2	13.4	6.5	7.8	7.2	10.2	11.0	20.0
Per cent of intake excreted (average)	55	40	34	33	43	35	40	25

[1] 2 mg riboflavin administered in a retention enema the morning of the eleventh day.

[2] First day excluded in estimation of per cent variation.

who consumed 2.5 to 2.7 mg riboflavin per day. The amount of vitamin excreted by subject GE agreed with the lowest values reported by Melnick and others.

The data in general indicated that all 8 women were suitable subjects for participation in this particular type of metabolic study.

It may be of interest to workers in other laboratories to know that the use of a uniform water intake did not assure a uniform output of urine. Since these studies extended over several months, wide differences were nevertheless encountered in weather conditions which greatly altered perspiration losses. It was noted, also, that the urine volumes were smaller just preceding menstruation although no changes were observed in the quantity of riboflavin excreted at these times. From the studies of Johnson ('46) it would seem that the excretion of riboflavin was not influenced by the volume of urine unless extremely small or excessively large amounts of urine were eliminated.

Influence of administration of riboflavin by retention enema upon the urinary excretion of the vitamin

Considerable interest has been aroused by the possibility that the amount of riboflavin excreted in the urine may be influenced by the synthesis of riboflavin in the intestinal tract. Data reported by Najjar and associates ('44) support this idea. A more recent investigation by Alexander and Landwehr ('45) on the role of fecal thiamine in human nutrition indicates that this vitamin is not absorbed from the lower portion of the intestine and that the urinary excretion of the vitamin does not change if physiological amounts of thiamine are administered by retention enema.

Since it was possible that the ingestion of the various test foods might alter the intestinal flora and in turn influence the urinary excretion of riboflavin due to absorption of the vitamins synthesized by the flora, the effect of administering physiological amounts of riboflavin by retention enema upon the urinary output of the vitamin was studied. Two mg of riboflavin were given by retention enema on the morning of the eleventh day of period I. The vitamin was retained by the various subjects from 10.5 to 39 hours and in no case was there a measurable influence on the excretion of riboflavin in the urine (table 4).

The importance of bacterial synthesis in providing available riboflavin to human subjects is therefore questioned. Although the introduction of test foods into the diet may have altered the total riboflavin synthesized in the intestinal tract, it would appear from the findings of this study that this vitamin was not absorbed.

Influence of adding 1 mg of pure riboflavin

In the availability studies reported by others (Melnick et al., '45; Oser, Melnick and Hochberg, '45), in which vitamin concentrates or the pure chemical were tested, larger supplements were used than is practical when foods supply the vitamin. It was necessary, therefore, to determine whether a smaller supplement would create sufficient rise in riboflavin excretion in the urine to constitute a reliable test of differences in availability. A dose of 1 mg was chosen for testing the response of the subjects, since quantities of foods required to supply this amount of the vitamin could be consumed without difficulty. To avoid the need for eating large amounts of food at one time as well as to make use of the possibility that a divided dose of riboflavin might permit more efficient absorption of the vitamin, the supplement was divided into 2 equal portions.

Data given in tables 5 and 6 show that the increment in the urinary excretion of the vitamin induced by the supplementation of the diet with riboflavin was surprisingly high. The per cent of the test dose returned varied widely (31 to 67%) in the different subjects. The average return for the 8 subjects was slightly over 42%. This percentage return was not much different from that observed by Melnick and associates for men ingesting 10 mg of riboflavin immediately after the noon meal.

Thus it appears that adequate stimulation of riboflavin excretion was produced by the addition of two 500 μg supplements, and that the technic was practical for testing the relative availability of riboflavin from food sources. The

effect upon the efficiency of absorption of ingesting frequent small amounts of riboflavin rather than 1 large supplement is being studied at the present time.

To check further the acceptability of the 1 mg quantity of riboflavin as the test dose, subject GE repeated the procedure outlined for period II during 4 consecutive months. Each test included the standardized preliminary period and the same basal diet. It may be noted from table 5 that this subject responded very uniformly, the per cent of the test dose returned ranging only from 32 to 38.

TABLE 5

Reproducibility of urinary excretion of riboflavin following supplementation of 1 mg pure riboflavin (subject GE).

PERIOD	RIBOFLAVIN SUPPLEMENT	BASAL EXCRETION	AFTER TEST DOSE	TEST DOSE RETURNED
	mg	*µg*	*µg*	%
I (December)	1	484	839	36
II (January)	1	593	917	32
III (February)	1	538	913	38
IV (March)	1	646	1017	37

Availability of riboflavin of ice cream, green peas, and almonds

When ice cream served as the test food a marked rise occurred in the quantity of vitamin appearing in the urine (table 6). The increment corresponded closely to that produced by the addition of 1 mg of the pure vitamin. Six of the 8 subjects responded similarly to the addition of riboflavin as the pure vitamin or as the test food, while 2 subjects (SK and GB) showed a slightly smaller excretion of the vitamin when riboflavin was derived from the food. The average percentage of test dose returned by the 8 subjects (38.5)

indicated that the riboflavin of ice cream was present in a physiologically available form.

When either frozen green peas or almonds furnished the 1 mg supplement, all 8 subjects excreted less riboflavin than they had on 2 previous occasions when either the pure vitamin or ice cream served as the source of the nutrient. These differences in excretion of the vitamin indicated that a smaller percentage of the total riboflavin occurring in peas or almonds was available.

TABLE 6

Availability of riboflavin of 3 foods.

SUBJECT	PERIOD II 1 MG RIBOFLAVIN (PURE VITAMIN)	PERIOD III 1 MG RIBOFLAVIN (ICE CREAM)	PERIOD IV 1 MG RIBOFLAVIN (GREEN PEAS)	PERIOD V 1 MG RIBOFLAVIN (ALMONDS)
	Per cent test dose excreted			
HW	39	33	20	5
HB	46	47	20	6
RM	43	43	18	15
SK	67	56	32	40
EW	38	34	20	22
GB	45	35	11	21
MC	31	28	9	3
GE	32	32	12	20
Average	42.6	38.5	17.7	16.5

Availability of riboflavin in ice cream $= \frac{38.5}{42.6} \times 100 = 90.4 \pm 11\%$

Availability of riboflavin in green peas $= \frac{17.7}{42.6} \times 100 = 41.5 \pm 11\%$

Availability of riboflavin in almonds $= \frac{16.5}{42.6} \times 100 = 38.7 \pm 11\%$

It should be brought out, however, that had the peas or almonds been used in another form, i.e., as the puréed vegetable or as almond paste, a higher percentage of the riboflavin present might have been absorbed.

While considerable individual difference was observed between subjects as to the percentage of test dose returned when 1 mg of pure vitamin was ingested, the subjects were fairly consistent in their response to the addition of the

vitamin in the form of food. It is interesting to note that SK, who excreted 67% of the test supplement when pure riboflavin was consumed always excreted the highest percentage of the test dose of any of the subjects regardless of the source of the vitamin, and that MC consistently excreted the smallest fraction of the added riboflavin.

No explanation can be given at this time for the striking differences in the apparent availability of riboflavin in the 3 food sources. It is thought that these differences may be due to variations in absorbability. Possibly the presence of bound forms of the vitamin in peas or in almonds may account for part of the difference. It may be found that the presence of certain other constituents in the intestinal tract at the time that riboflavin absorption takes place limits the quantity of the vitamin which is available. Apparently these differences cannot be explained by variations in acid-base balance since Johnson ('46) observed no change in urinary output of riboflavin following wide fluctuations in urinary pH.

Additional studies are planned to determine some of the factors which may influence the availability of riboflavin of foods.

SUMMARY

Eight healthy women subjects participated in 5 experimental studies in which the relative availability of the riboflavin present in ice cream, frozen green peas, and almonds was determined. During a 12-day period when a weighed diet containing 2.4 mg of riboflavin was ingested, the urinary excretion of riboflavin was strikingly constant.

Administration of 2 mg of riboflavin by retention enema on the morning of the eleventh day produced no change in the amount of riboflavin which appeared in the urine.

A marked rise in the concentration of riboflavin of the urine occurred for all subjects when two 500 μg portions of pure riboflavin supplemented the basal diet. The percentage of the test dose returned varied from 31 to 67 with an average value of 42% for the 8 subjects.

When a supplement of 1 mg of riboflavin was supplied in the form of ice cream, the increment in urinary riboflavin was comparable to that produced by the pure vitamin, indicating that the riboflavin of ice cream was nearly as available as that of the pure vitamin.

When either frozen green peas or almonds furnished 1 mg of the supplementary vitamin, considerably less riboflavin was excreted in the urine than when the pure vitamin was administered. Less than one-half as much riboflavin appeared to be absorbed from these foods as had been absorbed from ice cream or the pure vitamin.

Investigations are under way to determine some of the factors which influence the availability of riboflavin in foods.

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COMPARISON OF VITAMIN A LIVER STORAGE FOLLOWING ADMINISTRATION OF VITAMIN A IN OILY AND AQUEOUS MEDIA

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It has been previously observed in this laboratory that vitamin A when administered in aqueous media is absorbed much more readily than when administered in oil. This difference is especially important in conditions where impaired intestinal absorption exists, as in the celiac syndrome, and in the normal newborn infant (Kramer, Sobel and Gottfried, '47; unpublished results of Sobel, Besman and Kramer).[1] The criterion in absorption was the change in vitamin A blood serum levels following the administration of the vitamin A-containing preparation (Kramer, Sobel and Gottfried, '47; Sobel, Besman and Kramer[1]; May, Blackfan, McCreary and Allen, '40).

The question arose as to whether or not an increase in the blood level actually represents increased absorption. Since metabolic studies are open to the objection that vitamin A may be destroyed in its passage through the intestine itself (Sherman, '47), we undertook to determine vitamin A absorption by the liver storage test.

[1] Presented before the Baskerville Chemical Society of the College of the City of New York, April 24, 1947; before the New York Pediatric Research Club, New York City, April 2, 1947; and in part before the American Society of Biological Chemists, Chicago, May 22, 1947.

EXPERIMENTAL

The liver storage test was employed using essentially the technique of Lemley, Brown, Bird and Emmett ('47). The rats used were from an inbred Wistar strain kept on the stock diet of Bills et al. ('31). Fifteen-day-old rats, together with the mother, were put on the U.S.P. XII Vitamin A Test Diet [2] with the following percentage composition: vitamin-free casein 18, U.S.P. no. 2 salt mixture 4, irradiated yeast 8, starch 65, and hydrogenated vegetable oil mixture consisting primarily of hydrogenated cottonseed oil [3] 5. At 20 days of age, the weanling rats were removed to experimental cages and the U.S.P. diet and distilled water given ad libitum. On this regime deficiency followed in 25–30 days, as determined by failure to gain weight for 3 successive days or by the presence of xerophthalmia.

Following the vitamin A depletion period, each litter was split into groups, each group receiving one of the preparations which were being used for comparison. The vitamin A-containing preparations were administered on 3 successive days, 0.2 ml being given each time through a stomach tube, employing the technique of Shay and Gruenstein ('46). The stomach tube is essential since the rats will not take aqueous dispersions by simple oral feeding. On all preparations administered, the vitamin A content was determined at the beginning and at the end of each experiment, employing the method of Oser et al. ('43).

At the beginning of the fifth day, the rats were sacrificed by cutting the carotid arteries and jugular veins. The vitamin A content of the blood serum was determined by the GDH (activated glycerol dichlorohydrin) method of Sobel and Snow ('47). Each whole liver was analyzed separately, immediately after removal.

The method of liver analysis was simplified in order to shorten the procedure and to increase the reliability of results. The conventional method of analysis, as exemplified by Oser

[2] Obtained from General Biochemicals, Inc.
[3] Primex.

et al. ('43), requires a total of 10 extractions and washings in separatory funnels, and results in a large volume of extract. It also requires the preparation of freshly redistilled ethyl ether. The method of Gallup and Hoeffer ('46) where a 1-gm sample is punched out of the liver as an aliquot, is open to 2 objections: (1) the assumption of a representative sample is invalid (Sanford and Bucher, '46), and (2) it is difficult to remove and weigh a 1-gm sample without loss of weight by evaporation. In addition the 1 extraction with petroleum ether recommended by Gallup and Hoeffer ('46) may sometimes be incomplete.

In our analytical procedure each whole liver was finely minced and placed in a flask with 0.75 ml 60% KOH and 7.5 ml 95% aldehyde-free ethyl alcohol per gram of liver. The liver saponification mixture was refluxed on a hot water bath for 1 hour, or until the liver was completely dissolved, using ground-glass connections throughout. The resulting solutions were made up to 50 or 100 ml volume with 95% aldehyde-free ethyl alcohol in volumetric flasks, depending upon the original size of the liver.

From these solutions 1 ml aliquots were removed and placed in a glass-stoppered test tube, 1 ml of distilled water was added and the mixture extracted while being agitated by a shaking machine with 2 ml redistilled petroleum ether (B.P. 30°–60°C.) for 10 minutes. The tubes were then centrifuged at low speed for 30 seconds and the supernatant liquid removed by aspirating with a fine-tipped dropper. The extraction was repeated 2 more times using 1 ml of petroleum ether each time. The vitamin A content of the combined extracts was determined as described by Kramer et al. ('47) for the petroleum ether extract of blood serum. By this method as many as 10 simultaneous determinations may be completed by 1 person in 2 hours following saponification. The proposed method represents a step towards greater precision and reliability in single rat liver analysis, since duplicates and recoveries may be obtained, as contrasted to the methods where the whole liver is used for a single analysis. As shown in table 1 good agreement is demonstrated between this method

and that of Oser et al. ('43) which was carried out on 20 to 40 ml of digest representing 40% of the liver.

TABLE 1

Comparison of liver vitamin A analysis by the proposed method and by the method of Oser et al. ('43).

LIVER NO.	PROPOSED METHOD [1]	METHOD OF OSER ET AL.[1]	LIVER NO.	PROPOSED METHOD [1]	METHOD OF OSER ET AL.[1]
1	130	123	8	334	336
2	148	138	9	284	295
3	209	217	10	152	153
4	395	410	11	210	232
5	356	366	12	249	264
6	405	388	13	159	173
7	379	398			
			Mean value	262	269

[1] Expressed as total USP units of vitamin A per whole liver.

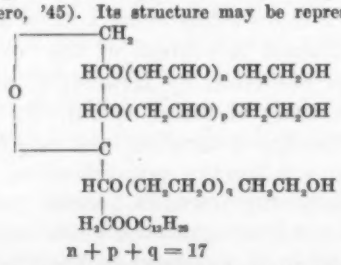
VITAMIN A ABSORPTION AS MEASURED BY LIVER STORAGE

Unsaponifiable fraction of fish liver oil in oily and aqueous media

In these experiments, a commercial concentrate [4] was used. This is the unsaponifiable fraction of fish liver oil containing about 800,000 to 1,000,000 U.S.P. units of vitamin A per gram. This material was diluted to the same degree (a) in maize oil, (b) in water containing 16% of the dispersing agent [5] sup-

[4] The Borden Co., New York.

[5] The dispersing agent used was sorbitan monolaurate polyoxyethylene derivative (Griffin, '45; Cordero, '45). Its structure may be represented by



$n + p + q = 17$

This compound is an edible dispersing agent. A full description of its physical, chemical and physiological properties may be obtained from the Atlas Powder Company under the name of Tween 20.

plemented by other vitamins to prepare aqueous dispersion I,[6] and (c) in water containing 16% of the dispersing agent to prepare aqueous dispersion II.[7]

The results are shown in table 2 and summarized in table 3. There was about 3 times as much vitamin A in the livers of the animals receiving aqueous dispersions I and II as in those receiving the oily solution. The difference between the deposition effected by the 2 aqueous dispersions is negligible, that due to aqueous dispersion I being slightly higher. Thus, under these conditions, as measured by the vitamin A storage test, this vitamin is far better absorbed from aqueous media than from an oily solution. There was no obvious relationship between serum vitamin A levels and liver storage, except that animals deficient in this vitamin (negative controls) showed no measurable vitamin A in the serum. This lack of correlation is in agreement with the findings of several other investigators (McCoord and Luce-Clausen, '34; Nylund and With, '41; Leong, '41). Glover, Goodwin and Morton ('47) "showed that the plasma vitamin A levels are proportional to the concentrations of vitamin A alcohol in the livers, but are not proportional to the total liver store of vitamin A which consists mainly of esters."

Unsaponifiable fraction of fish liver oil and natural esters in maize oil

The notable difference between the findings of the preceding experiment and those of Lemley, Brown, Bird and Emmett ('47) is that our percentage of vitamin A deposited in the liver in the case of the oily solution is only about one-third

[6] Aqueous dispersion I is called "Vifort." Each 0.6 ml contained in addition to the vitamin A, 1200 USP units of vitamin D, 1.8 mg of thiamine hydrochloride, 0.6 mg riboflavin, 0.3 mg pyridoxine hydrochloride, 60 mg of ascorbic acid, 3 mg of nicotinamide, 1.2 mg of calcium pantothenate and 132 mg of extract of rice polishings, and also contained choline, inositol and biotin vitamers.

[7] Aqueous dispersions I and II were similar to that previously used in children (Kramer, Sobel and Gottfried, '47) except that both contained 16% of dispersing agent instead of 11% in dispersion I and 20% in dispersion II. In the vitamin A absorption studies on newborn children (Sobel, Besman and Kramer, unpublished) both preparations contained 16% of the dispersing agent.

TABLE 2

Comparison of vitamin A liver storage in rats given vitamin A in oily and aqueous media.

Vitamin A values are expressed in USP units

MEDIUM	GROUP	NO. CASES	MEAN BODY WT.	AMT. VIT. A ADMIN.	AMT. ADM. PER GM WT.	MEAN LIVER DEPOSIT	% OF TOTAL DEPOSIT	SERUM VIT. A /100 ML
Vitamin A alcohol (Borden's concentrate) in maize oil and in aqueous dispersing media I and II								
Maize oil	A[1]	8	76.8	1250	16.3	123	9.8	75
	B	4	106.1	1090	10.3	129	11.8	98
Aqueous dispersion I	A	10	70.6	1250	17.7	355	28.4	88
	B	5	98.6	1155	11.7	362	31.4	99
Aqueous dispersion II	A	8	93.9	1390	14.8	385	27.7	123
	B	5	109.7	1275	11.6	366	28.8	87
Negative controls	A	3	109.3	....	...	10	...	0
Vitamin A alcohol (Borden's concentrate) and esters (Oleum Percomorph and distilled) in maize oil								
Vitamin A alcohol in maize oil	C[1]	2	88.0	1250	14.2	152	12.2	89
	D	3	57.8	1350	23.4	141	10.4	154
	E	3	61.7	2695	43.7	93	3.5	169
Oleum percomorph in maize oil	C	3	70.0	1250	17.9	124	9.9	66
Distilled ester in maize oil	D	3	51.0	1430	28.1	231	16.2	177
	E	3	62.0	2860	46.1	331	11.6	128
Vitamin A ester (distilled) in maize oil and in aqueous dispersing medium III								
Maize oil	F[1]	4	95.4	1215	12.7	217	17.9	246
	G	4	126.9	1220	9.6	160	13.1	111
Dispersion III	F	4	83.9	1215	14.5	465	38.3	166
	G	4	119.5	1195	10.0	349	29.2	103
Negative control	G	1	100.5	....	...	49	...	0

[1] Each letter represents corresponding litter groups.

that of the percentages reported by Lemley. This presented the possibility that the unsaponifiable fraction of fish liver oil is more poorly absorbed than other forms of vitamin A, such as the natural ester used by Lemley, Brown, Bird and Emmett ('47).

It has been observed by several investigators (Emmett and Bird, '37; Gray, Hickman and Brown, '40) that there is a difference between the biological assay value of vitamin A in

TABLE 3

Comparative efficacy of various vitamin A preparations.

Vitamin A values are expressed in USP units

PREPARATION	NO. CASES	MEAN LIVER DEPOSIT	% OF TOTAL DEPOSIT	SERUM LEVELS/100 ML		
				Low	Medium	High
Unsap. fish oil in oil solution	17	131	10.7	41	92	169
Distilled ester in oil solution	11	200	15.7	79	152	287
Oleum percomorph in oil	3	124	9.9	49	62	89
Aqueous dispersion I	15	357	29.4	67	96	118
Aqueous dispersion II	13	378	28.1	62	96	188
Aqueous dispersion III	8	407	33.7	96	118	217
Negative controls	4	20	...	0	0	0

the free alcohol form and that of the esterified form. As shown in table 2, under the conditions of this experiment, there is a negligible difference between the efficacy of unsaponifiable fraction of fish liver oil concentrate and Oleum Percomorph at the same level of administration. However, it appears that the distilled natural ester is about 1.5 times as efficient as the unsaponifiable fraction of fish liver oil at this level. Litter E, although an atypical example because of a prolonged de-

ficiency period (35 days), also shows a definite difference between the deposition of the unsaponifiable fraction and that of the natural ester. However, the maximum deposition attained with the natural ester is still only one-half of that reported by Lemley, Brown, Bird and Emmett ('47).

Distilled natural esters in oily and aqueous media

In these experiments a distilled natural ester [8] containing about 400,000 U.S.P. units of vitamin A per gram and 0.5% tocopherols, was diluted to the same degree in maize oil and in water containing 40% of the dispersing agent [9] to prepare aqueous dispersion III. This high percentage of the dispersing agent was used in order to obtain a clear dispersion. The results are shown in table 2. There was about 2.2 times as much deposition with the aqueous dispersion as with the oily solution. In the case of the aqueous dispersion of the natural ester, the absolute percentage of deposition was actually higher than the maximum deposition reported by Lemley, Brown, Bird and Emmett ('47) for oily solutions under optimum conditions.

The absorption of vitamin A as measured by liver storage of the various preparations

The results of all experiments are summarized in table 3. It may be readily observed that the aqueous dispersions in general showed a far higher percentage of absorption as measured by liver deposition than the same vitamin A-containing preparations diluted in maize oil. Among the oils, the distilled esters gave higher per cent deposition than the unsaponifiable fraction of fish oil.

DISCUSSION

The present experiments show that, in addition to those factors mentioned by Clausen ('43) as affecting the ability

[8] No. 11537, from Distillation Products, Inc.

[9] See footnote 5, page 228.

to absorb vitamin A, the medium of aqueous dispersion must also be considered an important factor. The probable explanation is that the size of the individual vitamin A-containing particles is in itself an important factor in absorption. The importance of particle size was indicated by Frazer et al. ('44). They have shown that olive oil in the intestine of the rat is dispersed to a fine particle size less than 0.5 μ. Furthermore, in rats fed paraffin in an aqueous dispersion containing particles of an average size of less than 0.5 μ, the paraffin is absorbed by the intestine as well as olive oil dispersed to an equal particle size in water, whereas *paraffin itself* is not absorbed at all by the intestine. Lundbaek and Maaløe ('47) however, were unable to repeat the paraffin experiments.

It is possible, in addition to the particle size property, that the dispersing agent itself plays a part in improved absorption by acting as a wetting agent, changing the inter-facial tension or changing the surface of the intestine or the channels through which the vitamin A is absorbed. In a paper published during the course of this investigation (Halpern, Biely and Hardy, '47) where vitamin A was dispersed in water by another agent (1% "methocel," 0.05% mixed tocopherols and 0.1% lecithin), it was shown by the bio-assay value as measured by the growth of chicks, that the aqueous dispersion was far more effective than the oily solution.

Polskin ('40) has shown that in the chick there is increased liver deposition when lecithin is added to the vitamin A supplement, while Slanetz and Scharf ('45) have shown the same effect for the rat. These effects may be due to the well-known emulsifying property of lecithin rather than to some specific effect of lecithin *per se*.

In view of these findings one can state with fair assurance that the sharp rise in the vitamin A tolerance curve for blood serum for aqueous dispersions, as opposed to the flat curves obtained with oily solutions (Kramer, Sobel and Gottfried, '47; unpublished results of Sobel, Besman and Kramer [10])

[10] See footnote 1, page 225.

(in cases of impaired absorption such as the celiac syndrome and the normal newborn infant), actually represents improved absorption and retention of vitamin A rather than merely a temporary high blood level.

Until all of the vitamin A administered is accounted for, it is difficult to completely eliminate stability, sparing action or both as factors. Such an explanation, however, would hardly account for an average maximum rise in serum vitamin A of 69 μg % with oil and 636 μg % with the same amount of vitamin A in water [11] (Kramer, Sobel and Gottfried, '47). Furthermore, there is no evidence to indicate that aqueous dispersions of vitamin A are more stable than vitamin A in oils. Fish oils and distilled esters contain antioxidants and are considered more stable than the nonsaponifiable fraction of fish oil present in dispersion II. It is not likely that a sparing action of vitamin A could be demonstrated in the short period of these experiments. Lemley, Brown and Emmett ('47) have found no significant sparing action of vitamin E on liver storage under experimental conditions similar to ours. They were able to demonstrate a sparing action only when smaller daily doses were fed for 3 to 6 months.

The vitamin supplements in aqueous dispersion I [12] did not reduce the amount of liver storage as would be expected from the vitamin A tolerance tests in normal children reported by Kramer, Sobel and Gottfried ('47). However, it was later shown in unpublished experiments by Sobel, Besman and Kramer that when the per cent of dispersing agent [13] is identical in aqueous dispersions I and II [12, 14] (as was the case in these experiments) the improvement in vitamin A

[11] Vitamin A tolerances obtained with vitamin A as the alcohol in oil (Borden's concentrate in maize oil) and vitamin A as the ester in oil (Oleum Percomorph) were similar (unpublished results of Sobel, Besman and Kramer). Thus the differences between vitamin A tolerances obtained in aqueous and oily media are not due to the fact that the ester was given in oil and the alcohol in aqueous medium as suggested by Embree ('47).

[12] See footnote 6, page 229.

[13] See footnote 5, page 228.

[14] See footnote 7, page 229.

absorption when compared to oily solutions (as measured by the vitamin A tolerance test) is the same with both aqueous preparations.

The results of this experiment show a markedly lower percentage of liver deposition in the case of oily preparations than that reported by Lemley, Brown, Bird and Emmett ('47). This may be explained by one or more of several factors known to affect the degree of absorption in experiments of this type. Several investigators have reported that the type and condition of the oil used in the basic deficient diet have a marked effect on the bioassay of vitamin A (Lathbury and Greenwood, '34; Lease et al., '38; Harrelson and coworkers, '39). Foy and Morgareidge ('47) in testing the Guggenheim and Koch ('44) method of bio-assay found that when vitamin A was dissolved in sardine oil, the deposition was 50% of that which they obtained when the vitamin A was dissolved in maize oil. The oil used in their vitamin A deficient diet was maize oil instead of the hydrogenated cottonseed oil in our diet. It is conceivable that if they had used sardine oil in their vitamin A deficient diet, the sardine oil effect would have been even more marked. Another factor involved is the actual nature of the vitamin A deficient diet (Gridgeman, Lees and Wilkinson, '40).

The well-known effect of vitamin E on vitamin A absorption and storage has been repeatedly emphasized. The subject has been reviewed by Moore ('45).

It is also possible that the difference between our results and those reported by Lemley, Brown, Bird and Emmett ('47) may be due to the difference in the strain of rats used rather than to the dietary factors mentioned above.

Regardless of the underlying cause of the discrepancy, our rats had impaired absorption of vitamin A from oil as compared to the rats used by Lemley, Brown, Bird and Emmett ('47). Thus, the situation in some respects is analogous to that of children with celiac syndrome who have impaired absorption as compared to normal children (Kramer, Sobel and Gottfried, '47) and newborn infants who have impaired ab-

sorption as compared to older children (unpublished data of Sobel, Besman and Kramer). However, the important fact is that, on administration of aqueous dispersions of vitamin A, we obtained greater absorption, as measured by liver storage, than with oily solutions. This same difference in absorption from oily and aqueous media was also observed in children with impaired absorption as measured by the vitamin A tolerance test.

A salient point brought out by this observation is the variability of the results which may be obtained by using the liver storage bio-assay procedure proposed by Guggenheim and Koch ('44), depending upon the nature of the medium used for the administration of vitamin A.

SUMMARY

Vitamin A liver storage tests were used as the criterion for determining the relative absorption of vitamin A from oily and aqueous media. Young rats were placed on the U.S.P. Vitamin A Test Diet containing hydrogenated cottonseed oil. The deficient rats were fed the preparations for 3 successive days and sacrificed at the beginning of the fifth day for vitamin A analyses of liver and serum.

There was no definite relationship between serum levels and liver storage, except that vitamin A deficient animals gave low serum values.

Liver storage was 3 times as great in groups fed the unsaponifiable fraction of fish liver oil dispersed in water as compared with the same fraction administered in maize oil.

Liver storage of groups fed distilled natural esters in maize oil was about 1.5 times as great as that deposited in groups fed the unsaponifiable fraction in maize oil.

Distilled esters dispersed in water gave 2.2 times the deposit as compared to distilled esters in maize oil.

Apparently vitamin A is more effective when dispersed in aqueous media than in oily solutions. This indicates the importance of considering the nature of the diluent material in the biological evaluation of vitamin A.

ACKNOWLEDGMENT

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NIACIN EXCRETION IN THE RAT IN RELATION TO TRYPTOPHANE, PYRIDOXINE, AND PROTEIN CONTENT OF THE DIET [1]

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A considerable body of evidence has accumulated in recent years to indicate that the formation of niacin in the organism of the rat is dependent on the intake of tryptophane (Rosen, Huff and Perlzweig, '46; Singal et al., '46). The most probable explanation of this relationship is that niacin is actually synthesized from tryptophane. On the other hand, beginning with the work of Lepkovsky and coworkers ('43), it has become increasingly evident that pyridoxine is intimately concerned in the metabolism of tryptophane. We accordingly began a study of the influence of pyridoxine in the diet on the niacin excretion of rats. While this work was in progress, Rosen, Huff and Perlzweig ('47) and Schweigert and Pearson ('47) published the results of studies showing that excretion of niacin and N^1-methylnicotinamide by rats, following administration of tryptophane, was greater in animals fed pyridoxine than in animals on diets deficient in this vitamin.

The results of our studies are in agreement with these conclusions, but show in addition that the effects of pyridoxine are more evident when the tryptophane is administered as the free amino acid. Singal and coworkers ('46) showed that an increase in the protein (casein) content of the diet did not increase niacin excretions; our results confirm this.

[1] The data are taken from a thesis presented by Grace H. Bell to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

METHODS

Rats of the University of Southern California strain were used. They were kept in large wire cages with screen bottoms or, for collection of urine, in individual metabolism cages. Urine was collected under toluene, or in bottles containing 3% acetic acid in amounts such that the final acetic acid concentration of urine and washings was about 2%. The preserved urines were stored in a refrigerator at 4°C. until analyzed. Niacin was determined on individual urines by microbiological assay with *Lactobacillus arabinosus* (U. S. Pharmacopoeia XII, first supplement revised), without acid hydrolysis. N^1-methylnicotinamide was determined fluorometrically by the method of Huff and Perlzweig ('47), using aliquots of pooled specimens from 5 rats.

The basal diet used in all experiments had the following composition: protein 15%, sucrose 72%, celluflour 4%, salts (Osborne and Mendel, '17) 4%, cottonseed oil [2] 3%, vitamin A and D oil mixture (6 parts cottonseed oil to 1 of superbiotol) 2%, choline chloride 150 mg, thiamine hydrochloride 2 mg, calcium pantothenate 4 mg, riboflavin 2 mg, and folic acid 0.5 mg per 100 gm diet. The proteins used were fish protein (Deuel et al., '45), vitamin-test casein,[3] and zein [4]. The fish protein was assayed for niacin and pyridoxine (Bird et al., '42), and no detectable amount of either was found. The zein, after acid hydrolysis, contained 0.6 mg of niacin and 0.3 mg of pyridoxine per 100 gm. To some diets, either protein or amino acids were added in excess of 15%; the sucrose percentage was decreased accordingly in these cases.

Niacin excretion on diets without added tryptophane

Ten male rats, weighing between 106 and 139 gm were fed the basal ration, deficient in pyridoxine, with 15% fish protein, for 2 weeks. They were then paired as to weight and food

[2] Wesson.

[3] General Biochemicals, Inc.

[4] Corn Products, Inc.

consumption. One member of each pair was continued on the basal ration; the other received the basal ration supplemented with 0.15 mg pyridoxine hydrochloride per 100 gm for the first week, 0.3 mg the second week, and 4.0 mg thereafter. The food intake of the animals fed pyridoxine was limited to that taken by the member of the pair on the ration lacking pyridoxine.

TABLE 1

Food intake, weight changes, and niacin excretion of rats fed the basal diet with fish protein, with and without pyridoxine. Average values for 5 rats in each group.

	GROUP [1]	TIME IN WEEKS					
		1	2	3	4	5	6
Pyridoxine intake,	+	0	0	14	26	405	362
μg/rat/day	—	0	0	0	0	0	0
Food intake,	+	12.3	10.2	9.6	8.7	10.0	9.0
gm/rat/day	—	12.3	9.9	9.3	9.0	10.0	9.0
Weight change,	+	+ 4.0	+ 2.4	— 0.5	+ 0.3	+ 3.2	+ 0.3
gm/rat/day	—	+ 2.9	+ 2.4	+ 0.2	+ 0.4	+ 2.0	+ 0.2
Niacin excretion,	+	42.6	46.0	56.3	42.6	43.8	53.6
μg/rat/day	—	29.6	37.5	43.5	32.2	37.5	37.5
N^1-methylnicotinamide excretion,	+	137.8	138.8	146.5	86.0	87.0	86.8
μg/rat/day	—	115.5	134.2	153.2	92.0	65.4	119.7
Total niacin [2] as	+	100	102.6	113.0	72.0	73.4	79
% of first week	—	100	118.2	135.7	86.0	72.2	107.9

[1] Group + received pyridoxine supplement during the third to sixth week of the experiment. The food intake of this group was restricted to that taken by group —, which received no pyridoxine.

[2] Niacin + N^1-methylnicotinamide.

The results of this experiment are presented in table 1. It is evident that the addition of pyridoxine to the diet does not result in an increased output of niacin or N^1-methylnicotinamide by rats maintained for 2 weeks on a diet lacking pyridoxine. The niacin excretion of the group receiving pyridoxine (+) was somewhat higher throughout than that of the deficient group (—), but there was no change in this

level when pyridoxine was added to the diet. No signs of pyridoxine deficiency, other than a decreased growth rate, reduced urine volume, and decreased activity, were noted in the pyridoxine-deficient group.

Niacin excretion on diets with varying tryptophane content

Twenty weanling rats, weighing 26–61 gm, were distributed among 4 large cages. The rats were all fed a basal ration containing 15% casein, and 0.2% L-cystine, with the composition otherwise as indicated earlier. The rats in 1 cage also received pyridoxine hydrochloride in the amount of 4 mg per 100 gm of ration; the basal ration contained no pyridoxine.

After 19 days on these rations, the rats were transferred to metabolism cages and the urine was collected during a 3-day period. On the twenty-second experimental day, the pyridoxine-deficient rats were divided into 3 groups. The first (− +) was given rations containing pyridoxine. The second (− −) was continued on the deficient diet. The third group (− + AL) was fed the ration with added pyridoxine ad libitum. The rats which had heretofore received pyridoxine (group + +) continued on the same ration, but their food intake was restricted to that of the first group (− +).

During the first period on this regime, the diet contained 15% casein (twenty-second to twenty-fifth day). L-tryptophane was then added in the proportion of 200 mg per 100 gm of diet (twenty-sixth to twenty-eighth day). In the third period (twenty-ninth to thirty-second day), the diet contained 30% casein, without added trytophane. There followed periods on 30% zein (thirty-third to thirty-sixth day), and zein with added tryptophane. After 42 days, the animals in groups − + and + + were returned to the original 15% casein diet, and the series of variations in tryptophane and protein intake were repeated with some modifications. The results of the entire series are presented in tables 2 and 3. The figures for total tryptophane intake were computed on the basis of a

TABLE 2

Food intake and niacin excretion of rats fed diets varying in pyridoxine, protein and tryptophane content. Average values for 5 rats in each group.

EXPERIMENTAL PERIOD:		1	2	3	4	5	6	7	8	9	10	11
DAYS:		20–22	23–25	26–28	29–32	33–36	37–39	40–42	43–46	47–48	49–51	52–53
Proteins and amino acids fed	Casein, %	15	15	15	30	..	..	..	15	15	..	3.8
	Zein, %	..	..	..	..	30	30	30	..	..	30	23.0
	L-tryptophane, mg %	..	..	200	..	..	135	335	..	200	335	305
Group ++ [1]	Food intake, gm/rat/day		7.2	4.3	7.5	5.0	5.0	5.0	12.5	10.0	7.2	10.0
	Total tryptophane intake, mg/rat/day		14.1	17.0	29.2	3.0	9.8	19.8	24.4	39.5	37.8	39.9
	Niacin excreted, μg/rat/day	14.6	19.3	50.0	42.5	14.6	29.3	29.3	15.6	28.4	90.0	64.0
	N^1-methylnicotinamide excreted, μg/rat/day	13.9	18.5	212.5	29.1	18.7	28.1	50.5	6.3	163.0	63.6	46.6
−+ [2]	Food intake, gm/rat/day		7.2	4.3	7.5	5.0	5.0	5.0	12.1	10.0	7.4	10.0
	Total tryptophane intake, mg/rat/day		14.1	17.0	29.2	3.0	9.8	19.8	23.6	39.5	38.0	39.9
	Niacin excreted, μg/rat/day	15.0	19.7	26.6	22.0	15.6	16.3	25.3	15.3	30.1	96.0	62.0
	N^1-methylnicotinamide excreted, μg/rat/day	26.3	20.8	126.2	37.4	20.0	51.3	63.5	14.5	91.7	86.6	26.1

[1] Group ++ fed pyridoxine throughout. Food intake restricted to that of group −+.
[2] Group −+ pyridoxine-deficient from first to twenty-second day, fed pyridoxine thereafter.

tryptophane content for casein of 1.3% (Greenhut et al., '46) and for zein, 0.2% (Schmidt, '38).

A number of important points appear in these tables. The most striking is probably the contrast between experimental periods 3 and 4. The excretion of niacin and methylnicotinamide was increased in all cases by the addition of L-tryptophane to the diet (period 3). When, however, the same amount

TABLE 3

Food intake, and niacin excretion of rats fed diets varying in pyridoxine, protein and tryptophane content. Average values for 5 rats in each group.

EXPERIMENTAL PERIOD:		1	2	3	4	5	6	7
DAYS:		20–22	23–25	26–28	29–32	33–36	37–39	40–42
Proteins and amino acids fed	Casein, %	15	15	15	30	..	..	..
	Zein, %	..	..	..	..	30	30	30
	L-tryptophane, mg %	..	..	200	..	..	135	335
Group —— [1]	Food intake, gm/rat/day		5.0	4.0	4.8	3.7	4.5	4.7
	Total tryptophane intake, mg/rat/day		9.8	15.8	18.7	2.2	9.2	18.6
	Niacin excreted, μg/rat/day	15.0	10.6	12.5	10.3	11.5	12.0	15.4
	N^1-methylnicotinamide excreted, μg/rat/day	26.3	17.1	25.0	15.7	13.0	29.9	64.3
— + [2] AL	Food intake, gm/rat/day		8.0	8.0	10.0	5.2	6.7	6.1
	Total tryptophane intake, mg/rat/day		15.6	31.6	39.0	3.1	13.0	24.2
	Niacin excreted, μg/rat/day	15.0	9.9	36.6	26.0	18.6	21.8	42.5
	N^1-methylnicotinamide excreted, μg/rat/day	26.3	5.3	112.3	62.9	41.4	26.6	67.6

[1] Pyridoxine-deficient throughout.

[2] Pyridoxine-deficient first to twenty-second day. Fed pyridoxine-containing diets ad libitum thereafter.

of tryptophane was added to the diet by doubling the casein content, the excretion of niacin and methylnicotinamide fell off very markedly. Evidently tryptophane, combined as casein, is not as effective in stimulating niacin synthesis as is free tryptophane. Moreover, in most instances, the addition of tryptophane to a diet containing zein has a less striking effect than similar additions to a casein diet.

In most cases, the response to added tryptophane appears as an increase in excretion of N^1-methylnicotinamide, not niacin. This is particularly evident in the animals fed diets lacking in pyridoxine (group — —, table 3). Here, the niacin excretion remained at the same low level throughout the experiment. Such small increases as were observed after tryptophane were entirely confined to the methylated fraction. In the groups fed diets lacking in pyridoxine during the preliminary period, and then supplemented with pyridoxine later (group — +, table 2), the level of excretion was higher and the response to added tryptophane greater; here again, however, the response was most evident in the methylated fraction. The only exception appeared in the last experimental periods (10 and 11, table 2), where a definite increase in niacin excretion was evident. In animals fed pyridoxine throughout (group + +, table 2), the effect of added tryptophane was evident in both fractions, but was greatest in the methylated niacin. Again, the last experimental periods (10 and 11) constituted an exception; there niacin excretion increased, with a decrease in excretion of N^1-methylnicotinamide.

DISCUSSION

There is no immediate explanation for all of the phenomena observed here. The relation of pyridoxine deficiency to niacin synthesis is a remarkable one; as Schweigert and Pearson ('47) have shown, animals on a diet lacking pyridoxine excrete less niacin after tryptophane feeding than animals fed pyridoxine. However, Rosen, Huff and Perlzweig ('47) report that addition of pyridoxine to a diet deficient in this

vitamin does not wholly restore the increased niacin excretion in response to tryptophane. Our results confirm both of these reports. The most obvious explanation for these phenomena would be that of synthesis by intestinal micro-organisms. In pyridoxine deficiency these symbionts would die out, and not be readily replaced on restoration of pyridoxine to the diet. However, the fact that increased excretion of niacin is observed after injection of tryptophane (Schweigert and Pearson, '47) suggests that the tissues of the rat are able to carry out the transformation. We are now investigating the effects of pyridoxine deficiency on the intestinal flora.

The facts that tryptophane, fed as casein, has little or no effect on niacin excretion, and that L-tryptophane, fed with zein, is less effective than is the same amino acid fed with casein, suggest that amino acids other than tryptophane are concerned in the formation of niacin. Krehl and coworkers ('46) have suggested that the poor growth of rats on corn diets low in niacin is the result of an amino acid antagonism in which glycine is an important factor. Amino acid antagonism has been reported frequently in the microbiological literature since Gordon and M'Leod ('26) showed inhibitory effects of several amino acids on growth of bacteria in protein digests. Sullivan, Hess and Sebrell ('32) and later workers have reported similar phenomena in animals.

Beadle, in a personal communication, has reported that, in *Neurospora*, tryptophane stimulates niacin synthesis, but is antagonized by leucine. This offers a possible explanation for the fact that tryptophane is less effective in the rat on a zein diet than on a diet containing casein; zein contains a much larger amount of leucine than does casein. Krehl et al. ('46) reported no inhibition in the growth of rats when leucine (1%) was added to a diet containing 9% of casein; no data were reported on niacin excretion. We are now examining the effect of leucine and other amino acids on the niacin excretion in response to tryptophane.

SUMMARY

1. Addition of pyridoxine to a ration lacking this vitamin does not increase the excretion of niacin or N^1-methylnicotinamide by the rat.

2. Rats fed rations deficient in pyridoxine do not excrete less niacin or N^1-methylnicotinamide than rats fed rations adequately supplemented with this vitamin.

3. Addition of L-tryptophane to an adequate ration results in a marked increase in the excretion of both niacin and N^1-methylnicotinamide.

4. Addition of L-tryptophane to a diet deficient in pyridoxine results only in a very small increase in excretion of N^1-methylnicotinamide; niacin excretion is unchanged.

5. When rats are maintained on a diet deficient in pyridoxine for 22 days, and the diet is subsequently supplemented with pyridoxine, the increase in excretion of niacin after tryptophane is small. The excretion of N^1-methylnicotinamide, however, increases greatly.

6. The increase in excretion of niacin and its methylated amide is greater when tryptophane is fed in a diet containing casein than in a diet containing zein as the protein.

7. Little or no increase in niacin or N^1-methylnicotinamide is observed when the tryptophane intake is increased by feeding a high-protein diet.

8. The significance of these results is discussed. It is concluded that pyridoxine is concerned in the action of tryptophane in increasing niacin excretion, and that other amino acids are also involved, perhaps as antagonists to tryptophane.

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MINERAL METABOLISM STUDIES IN DAIRY CATTLE

II. EFFECT OF CALCIUM AND MANGANESE AND OTHER TRACE ELEMENTS ON THE METABOLISM OF LIPIDS DURING EARLY LACTATION [1]

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The literature regarding the interrelationships of mineral and fat metabolism has emphasized for the most part the effect of lipids on Ca and P metabolism with the greatest emphasis being placed on the antirachitic effect of lipids.

Holt et al. ('20, '23) found that the utilization of Ca by infants was augmented by fat in the diet, and Hickmans ('24) essentially verified their results. An increased absorption of Ca and P from the intestines of rats was observed by Boyd, Crum and Lyman ('32) when fat was added to the diet.

Holt, Tidwell and Kirk ('33), studying the effect of mineral intake upon fat retention in infants, found that an inverse relationship existed between the level of mineral intake and the portion of fat intake which was retained, with fat retentions of 81.4 and 94.8% for "high ash" and "low ash" diets, respectively. In a study of the feces from normal breast-fed and bottle-fed infants, Nielsen ('43) found that the total fat content of the dry feces was nearly twice as high for

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bottle-fed infants while the total solids content was the same for both groups. He also noted that, in the breast-fed infants, fat was excreted chiefly in the form of free fatty acids and neutral fat and, to a considerably lesser degree, as soap, whereas in the bottle-fed infants the soap fraction in the dry feces averaged 78% of the total fat. The ash and CaO values of the dry feces were 8.94 and 1.86%, respectively, for the breast-fed infants and 23.45 and 9.50%, respectively, for the bottle-fed infants.

Williams et al. ('43), studying the fat partition of 19 healthy children from 4 to 12 years of age, found that excretion of free fatty acids, neutral fat and unsaponifiable material was approximately equal for all subjects but that soap excretion was significantly higher from children with larger fat intakes. For the group of children as a whole, soaps comprised approximately 50% of the total fecal fat; unsaponifiable fat represented 30%, and the remainder was almost equally divided between free fatty acid and neutral fat.

EXPERIMENTAL

The data reported in this paper were derived from the mineral balance trials given in paper I of this series (Reid et al., '47). Three weekly balance trials were conducted during the first 5 months of lactation at regular intervals with each of 8 Holstein and 4 Guernsey cows, comprising 3 collection and 4 feed groups.

The general feeding plan provided 1 pound of timothy-clover hay and 3.5 pounds of corn silage daily per 100 pounds body weight and a concentrate mixture, containing approximately 5% ether extractive material, in sufficient quantity to satisfy the Morrison ('40) feeding standard for maintenance and production plus 10%. Group I received the basal concentrate mixture; group II, basal concentrate mixture plus 3% of $CaCO_3$ (c.p.); group III, basal concentrate mixture plus 3% of a mixture of $CaCO_3$ (c.p.) and $MnSO_4$ (c.p.) (proportions of Ca and Mn same as for group IV); group IV, basal

concentrate mixture plus 3% of a trace-element-fortified Ca supplement.[2]

The official method of the Association of Official Agricultural Chemists ('40) was used to determine the ether extractive content of the concentrate mixtures, hay, silage and feces.

The lipid fractionation was performed by the method of Forbes and Atkinson ('43) with modifications (Ward and Reid, '47). The method employs acid hydrolysis, $CHCl_3$ as the lipid solvent, an artificial zeolite as the phospholipid adsorbent and dichromate oxidation as a means of lipid estimation. Neutral fat and sterols, free fatty acids and soaps, phospholipids and the total lipids were determined.

RESULTS

The results obtained from the ether extraction and lipid fractionation analyses of the concentrate mixtures, hay, silage and feces were used to calculate the percentage of the intake of each fraction which was excreted in the feces. The loss percentages and average daily calcium intakes, their means, the standard errors of the means and the statistical significance of the differences between the mean lipid losses are presented in table 1. The proportion of ingested ether extractives lost in the feces of animals not receiving supplemental Ca (group I) was highly significantly greater than in that of animals receiving rations supplemented with Ca (groups II, III and IV). Although there was no significant difference among the means of the total lipid losses for the various groups, a significantly greater proportion of the ingested neutral fat and sterols was excreted in the feces of animals not receiving supplemental Ca (group I) than was lost by animals receiving increased quantities of this element (groups II, III and IV). The greater loss of neutral fat and sterols from group I animals was compensated to some extent by a greater retention of soaps and free fatty acids by these Ca unsupplemented cows.

[2] Mico, having the following percentage composition: calcium 33, mangnesium 2, manganese 0.20, iron 0.20, iodine 0.045, copper 0.025, zinc 0.01 and cobalt 0.002.

Group III animals excreted a significantly greater proportion of the ingested soaps and free fatty acids than did those of groups I and II.

The results of this study point out the inadequacy of the ether extraction method as such for the measurement of total lipids in cow feces and particularly in the feces of cows receiving large quantities of Ca.

TABLE 1

Mean percentages by fractions of ingested lipids excreted in the feces and the mean daily calcium intakes.

GROUP NO.		DAILY CALCIUM INTAKE	FRACTION				
			Neutral fat and sterols [1]	Soaps and free fatty acids [2]	Phospholipids	Total lipids	Ether Extractives [3]
		gm	%	%	%	%	%
I	Mean [4]	47.97	44.68	24.34	50.55	32.88	23.57
	St'd error	3.46	2.83	1.75	9.60	2.27	2.00
II	Mean	99.23	34.74	26.49	34.72	29.77	12.17
	St'd error	4.68	2.43	1.21	5.90	1.16	1.16
III	Mean	97.17	36.08	30.55	33.51	32.42	14.76
	St'd error	6.20	2.36	1.58	4.71	1.68	0.85
IV	Mean	99.78	32.90	27.09	31.69	29.16	15.59
	St'd error	3.22	2.32	2.02	6.26	2.07	1.17

[1] Mean differences are significant at the 5% level of probability (Fisher, '37) for groups I and II, I and III and at the 1% level of probability for groups I and IV.

[2] Mean differences are significant at the 5% level of probability for groups I and III and II and III.

[3] Mean differences are significant at the 1% level of probability for groups I and II, I and III, and I and IV.

[4] Each mean represents 9 values obtained from 3 animals, each on trial 3 times.

DISCUSSION

It is notable that the mean loss of ingested total lipids via the feces was similar for the animals of all groups, whereas a highly significantly greater loss of the ingested ether extractives was found in the feces of the animals of group I, not receiving supplemental Ca. These data would indicate that

the utilization of total lipids by the lactating cow is not related to the Ca intake within the limits employed in this study; but, upon a comparison of the data dealing with ether extractives, it would follow that the extraction of some lipid materials by di-ethyl ether is precluded in a manner in some way related to the quantity of Ca ingested by the animal. Since soaps are not soluble in di-ethyl ether it is quite probable that they make up a large portion of the lipid material not accounted for in the feces by the ether extraction procedure. Because of this shortcoming of the usual ether extraction method, it is evident that the apparent digestibility of crude fat would be unjustifiably elevated when cows receive high Ca rations, as is shown by the data for ether extractives in table 1.

Since the total lipid excretion was of similar magnitude in all groups and since group I excreted the largest proportion of neutral fat and sterols and the smallest proportion of soaps and free fatty acids, it would seem to be indicated that the increased Ca intake was largely responsible for the sparing of neutral fat at the expense of soaps and free fatty acids.

Kon and Booth ('33) showed that fat had a sparing action on Ca as shown by its antirachitic effect. In view of the data presented here, it would seem that the statement can be reversed in the case of the lactating cow, i.e., that Ca has a sparing action on the neutral fat and sterol fraction, while the soaps and free fatty acids are simultaneously expended. In contrast to this, Holt et al. ('33) found that the lipid loss in the feces of infants was directly proportional to the ash content (largely made up of Ca) of the diet.

It is possible that other mineral elements in the diet affect the lipid retention but, under the conditions of this experiment and in the amounts of Mn, I, Fe, Cu, Zn and Co supplemented, only Mn when added singly to the calcium had a significant effect, and that was qualitative rather than quantitative.

SUMMARY

A study was made of the effects of Ca, Mn, and other mineral element supplementation upon the metabolism of lipids during the first 5 months of lactation of 8 Holstein and 4 Guernsey cows.

All groups excreted similar proportions of the total lipid intake, as measured by acid hydrolysis and $CHCl_3$ extraction, regardless of the mineral supplement received. Therefore the observed group differences relative to the smaller proportion of ether extractives lost in the feces of animals receiving supplemental Ca were attributed to an increased formation of soaps which were not measured in the feces of these animals because of the insolubility of soap in di-ethyl ether, and not to a more efficient lipid use. Although the ingestion of Ca tended to spare neutral fat and sterols, a corresponding increase was observed in the excretion of soaps and free fatty acids, resulting in a qualitative rather than a quantitative effect.

These data would indicate that the estimation of crude fat by the commonly employed ether extraction procedure is likely to be misleading in experiments involving the analysis of dairy cow feces.

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THE BIOLOGICAL VALUE OF THE PROTEIN OF FIELD PEA PRODUCTS WITH A COMPARISON OF SEVERAL METHODS USED FOR THIS DETERMINATION [1]

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Field peas (Pisum sativum) have been extensively employed as a source of protein in famine areas in Europe and Asia as well as in low cost dietaries in the United States. Cull peas provide a protein concentrate of high value for animal feeding. Woods et al. ('43) and Russell et al. ('46) working with rats, and Peterson and Lampman ('44) using chickens, demonstrated that a low methionine content was a limiting factor when peas were used as the sole source of protein in growth experiments. Lehrer et al. ('47) in growth trials with rats have shown that meat supplements overcome this deficiency.

An examination of the literature points to some controversies regarding adequate methods for measuring biological value of proteins. Mitchell ('44) showed that the ratio of body weight gain to protein eaten may be changed by increasing food intake. Thus, a large experimental error may result in this ratio when ad libitum feeding is employed, tending to favor the efficiency of the better protein which is usually consumed in larger amounts. Barnes and Bosshardt ('46)

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concluded that pair-feeding gives a low value for the better protein since, if restriction is severe, some of it may be wasted for fuel. These workers cite evidence to prove that for several different proteins when there is maximal utilization, the amount absorbed is the same. Therefore, by ad libitum feeding, protein intake may be equalized and the greatest utilization obtained.

The purpose of the experiments in Part I of this paper was to determine (a) the biological value of the protein of various pea products, both raw and cooked, and (b) the effect of supplementation with various cereals. The purpose of the work reported in Part II was to make a comparison of 3 methods used for the estimation of biological value, namely, protein efficiency and retention in pair-fed versus ad libitum trials and a metabolism study using diets consisting of 10% casein and raw pea protein.

PART I. THE BIOLOGICAL VALUE OF VARIOUS PEA PRODUCTS — THE EFFECT OF SUPPLEMENTATION AND HEAT ON PROTEIN EFFICIENCY

Experimental

The germ, the sprouts and the entire seed, both from immature and mature plants of the Alaska field pea were studied. The germ resulted from the splitting process and constituted approximately 40% of the mixture, the rest consisting of fragments of the cotyledon. Peas were allowed to germinate until the sprouts were 2 to 3 inches in length, then blanched and dehydrated. Immature peas were harvested at the canning or freezing stage, blanched and dehydrated. The mature seed was used as harvested. Canned peas were soaked overnight, parboiled, packed in no. 2½ cans, sealed and autoclaved for 40 minutes at 10 pounds pressure (240°F.), then dehydrated. Baked peas were prepared by soaking overnight, parboiling for 15 minutes, and then baking for 2 hours at 375°F.; the product was then dehydrated. All dried products were ground and then analyzed for moisture and nitrogen.

The quantity of each required to furnish a protein level (N × 6.25) of 10 or 15% was then used in making up the diets (table 1). Due to their high methionine content, cereals (wheat, corn and barley) were used as supplements in some of the diets.

Each rat received as a supplement 3 times a week a vitamin solution containing thiamine hydrochloride 50 μg, riboflavin 50 μg, pyridoxine 50 μg, calcium pantothenate 250 μg, nicotinic acid 580 μg, inositol 130 μg, choline hydrochloride 20 mg,

TABLE 1

Percentage composition of diets.

INGREDIENTS	WHOLE PEA	CASEIN	PEA SPROUT	PEA GERM	PEA [1] WHEAT	IMMATURE PEA	PEA BARLEY	PEA CORN	PEA [1] WHEAT
Whole pea	44.6	...	...	...	30.0	...	26.6	26.6	22.3
Casein	...	11.0	...	...	...	...	...	...	...
Pea sprouts	...	...	41.3	...	...	...	...	...	...
Pea germ	...	...	...	31.7	...	...	...	...	...
Whole wheat	...	...	...	...	37.0	...	...	...	46.3
Immature pea	...	...	...	...	...	36.2	...	...	...
Pearl barley	...	...	...	...	...	...	38.3	...	...
Yellow corn	...	...	...	...	...	...	...	40.0	...
Sugar or starch [2]	42.9	76.5	46.2	55.8	20.5	51.3	22.6	20.9	18.9
Hydrogenated fat	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Hubbell salts [3]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5

[1] This combination was tested twice, one containing less pea than the other.

[2] Starch was substituted for sugar in diets used in experiments 3 and 4 due to wartime shortage.

[3] Hubbell et al. ('37).

p-aminobenzoic acid 250 μg, vitamin A 330 I.U., vitamin D 33 I.U., and mixed tocopherols 2 mg.

Animals were paired as to sex and size, and placed on experiment when they weighed 40 to 60 gm. They were housed in individual cages and weighed 3 times weekly. The trials in experiments 1 and 2 and the group on casein in experiment 3 were run on animals pair-fed for equal food consumption; the food intake of animals on each diet was regulated to that of the one on the raw pea diet.

Results and discussion

Results of this part of the study are summarized in tables 2 and 3. When casein and pea protein were compared on the basis of protein efficiency (gain per gm of protein eaten), casein proved to be approximately 50% better. The germ appeared to be slightly but not significantly superior in quality to the whole pea. Both sprouting and harvesting at the immature stage significantly lowered protein efficiency. This is contrary to the work on soybeans of Everson et al. ('44), who found that the germinated and raw, immature beans were distinctly superior to the mature ones.

The addition of cereals (corn, wheat and barley) materially improved the efficiency of the pea protein. The 3 cereals when substituted at a 5% protein level gave quite comparable results, all of which showed significant improvement over the pea protein alone. Apparently supplementation below the 5% level is not adequate as shown by a lower value where wheat contributed only one-third of the protein. The improvement due to cereal protein can no doubt be attributed to the high methionine content of these grains, thus compensating somewhat for this amino acid deficiency in peas.

Woods et al. ('43), in their work on the biological value of the field pea, secured an average protein efficiency of 1.0, and Everson and Heckert ('44) obtained a figure of 0.76 for green peas and 0.63 for the yellow variety. Values reported by these workers are somewhat lower than the figure in this study but the difference in value between casein and raw pea was quite comparable in the 3 investigations. Comparison of the results in table 2 with those of previous reports is valid since growing rats of approximately the same weight were used in all studies.

Raising the level of pea protein to 15% produced significantly greater gains but did not alter the efficiency. These findings are in agreement with those of Woods et al. ('43) where they compared raw pea protein at the 10 and 20% level. However, Jones and Widness ('46) secured a lowered

TABLE 2

Growth response of rats on various diets used to determine biological value of pea protein.

DIETS	NO. OF ANIMALS	AVERAGE TOTAL GAIN	AVERAGE GAIN PER GRAM OF PROTEIN	AVERAGE FOOD REQUIRED PER GRAM OF GAIN	AVERAGE FOOD EATEN DAILY PER RAT
		gm	gm	gm	gm
Experiment [1] no. 1 (pair-fed)					
10% raw pea	10	66.5 ± 5.0 [2]	1.38 ± 0.06 [2]	6.58 ± 0.30 [2]	7.6
10% pea sprout	8	51.5 ± 2.0	1.23 ± 0.03	7.91 ± 0.16	7.3
10% raw pea germ	8	71.0 ± 4.4	1.58 ± 0.09	6.12 ± 0.33	7.7
3½% whole wheat 6⅔% raw pea	10	79.8 ± 5.8	1.58 ± 0.07	5.51 ± 0.36	7.6
Experiment [1] no. 2 (pair-fed)					
10% raw pea	10	52.5 ± 2.2	1.47 ± 0.06	6.60 ± 0.29	8.4
5% raw pea 5% whole yellow corn	10	70.7 ± 2.6	1.98 ± 0.09	4.91 ± 0.22	8.2
5% raw pea 5% pearl barley	10	71.1 ± 2.3	1.78 ± 0.06	4.91 ± 0.16	8.3
5% raw pea 5% whole wheat	10	66.5 ± 2.1	1.80 ± 0.05	5.11 ± 0.14	8.1
10% immature pea	10	42.5 ± 2.0	1.16 ± 0.05	8.09 ± 0.40	8.1
Experiment [1] no. 3 (pair-fed)					
10% casein	8	45.9 ± 2.9	1.97 ± 0.08	5.49 ± 0.68	5.9
10% raw pea	8	33.8 ± 1.5	1.36 ± 0.09	7.19 ± 0.31	5.8
10% baked pea	8	35.4 ± 2.3	1.06 ± 0.05	9.77 ± 0.49	8.0
10% canned pea	7	17.5 ± 1.5	0.71 ± 0.05	11.38 ± 0.95	6.0
Experiment [1] no. 4 (pair-fed)					
10% casein	9	70.1 ± 3.2	1.82 ± 0.08	5.60 ± 0.24	9.3
10% raw pea	9	49.1 ± 1.6	1.23 ± 0.06	8.18 ± 0.40	9.5
Ad libitum feeding					
10% casein	9	87.4 ± 4.2	2.05 ± 0.07	4.93 ± 0.15	10.0
10% raw pea	9	53.0 ± 2.2	1.34 ± 0.06	7.48 ± 0.31	9.3
15% raw pea	10	102.7 ± 3.6	1.33 ± 0.03	5.02 ± 0.13	12.2

[1] Duration of experiment 1 was 8 weeks; experiments 2, 3 and 4 ran for 6 weeks.
[2] Standard error of single observations.

TABLE 3

Statistical comparisons of growth on various diets.

DIETS COMPARED	GRAMS GAINED		GRAMS GAINED PER GRAM PROTEIN	
	Mean Difference	t [1]	Mean Difference	t [1]
	Experiment no. 1 (pair-fed)			
10% raw pea vs. 10% pea sprout	15.0 ± 5.4 [2]	2.77 [3]	0.15 ± 0.07 [2]	2.14 [3]
10% raw pea vs. 10% pea germ	4.5 ± 6.6	0.70	0.20 ± 0.10	2.00
10% raw pea vs. 6⅔% raw pea and 3⅓% whole wheat	13.3 ± 7.6	1.75	0.20 ± 0.09	2.22 [3]
	Experiment no. 2 (pair-fed)			
10% raw pea vs. 5% raw pea-5% corn	17.5 ± 3.4	5.15 [4]	0.51 ± 0.11	4.63 [3]
10% raw pea vs. 5% raw pea-5% barley	18.6 ± 3.2	5.81 [4]	0.31 ± 0.08	3.87 [3]
10% raw pea vs. 5% raw pea-5% wheat	14.0 ± 3.0	4.67 [4]	0.33 ± 0.08	4.11 [3]
10% raw pea vs. 10% immature pea	10.0 ± 2.9	3.45 [4]	0.31 ± 0.08	3.87 [3]
	Experiment no. 3 (pair-fed)			
10% raw pea vs. 10% canned pea	16.3 ± 2.1	7.76 [4]	0.65 ± 0.10	6.50 [4]
10% raw pea vs. 10% baked pea	1.6 ± 2.1	0.76	0.30 ± 0.10	3.00 [4]
	Experiment no. 4			
10% casein vs. 10% raw pea pair-fed	21.0 ± 3.5	6.00 [4]	0.59 ± 0.10	5.90 [4]
10% casein vs. 10% raw pea ad libitum fed	34.4 ± 4.7	7.32 [4]	0.71 ± 0.09	7.89 [4]
10% casein pair-fed vs. 10% casein ad libitum fed	17.3 ± 5.2	3.33 [4]	0.23 ± 0.13	1.77
10% raw pea vs. 15% raw pea	53.6 ± 3.9	13.73 [4]	0.10 ± 0.07	1.43

[1] Calculated from Fisher's formula (Snedecor, '38).
[2] Standard error.
[3] Denotes significance at the 5% level.
[4] Denotes significance at the 1% level.

efficiency in tests of a series of proteins of vegetable origin (wheat, corn germ, soybean, cottonseed and peanut flour) with increasing protein content of the diets, comparisons being made at a 10, 15 and 17.5% level.

Baking lowered the efficiency of the protein significantly, despite the fact that consumption of this diet was greater than that of the raw pea. Woods et al. ('43) found that supplementation of autoclaved pea protein with 0.5% cystine restored the efficiency to that of the raw pea. Evans and St. John ('47) report that losses amounting to 24% of the cystine in peas result from autoclaving for 60 minutes at 130°C., whereas no change takes place in the methionine content. Since the rat's requirement for sulfur-containing amino acids is high (Cox et al., '47) and the amount in peas is low, losses would quickly be registered in a decreased growth rate. This deficiency also causes the loss of guard hairs and the development of a soft, short fur after the animal has been on experiment for several weeks.

PART II. A COMPARISON OF METHODS FOR DETERMINING BIOLOGICAL VALUE OF PROTEINS

Experimental

In experiment 4 a comparison was made of the pair-fed versus ad libitum methods using 10% raw pea and 10% casein diets. The trials were conducted as were those in experiments 1, 2 and 3 in Part I, with the exception that 1 group of animals on the casein diet was fed ad libitum. At the conclusion of the experiment, animals were killed, the gastrointestinal tract removed and the ground carcass analyzed for moisture and protein. Protein storage was obtained by subtracting the protein content of a group of control animals. Nitrogen determinations were run by the macro Kjeldahl method.

The method of Mitchell ('24) and Mitchell and Carman ('26) was used for the metabolism study. The 10% casein and 10% raw pea diets were employed during the test period. The

standardizing diet contained 4% whole egg protein and sufficient additional fat to make the total 10% correspond to the fat content of the test diets. All diets contained 2% agar for bulk. Test periods were 10 days in duration and collections were made for the last 6 days. Three periods were run on the standardizing diet, 1 each for the test proteins.

Results and discussion

In order to compare the 2 methods of feeding (pair and ad libitum) the growth rate of animals on a 10% casein diet was used (tables 2 and 3). A significant difference was observed in their weight gains but not in protein efficiency.

TABLE 4

Comparison of methods for determination of biological value. Pair-feeding versus ad libitum feeding.

	NO. OF ANIMALS	DIET	TOTAL PROTEIN STORED	PROTEIN STORED PER GRAM OF PROTEIN EATEN	MOISTURE
			gm	*gm*	%
Pair-fed	9	10% casein	15.00 ± 0.70 [1]	0.39 ± 0.02 [1]	62.75 ± 1.06 [1]
	9	10% raw pea	9.37 ± 0.48	0.25 ± 0.01	63.30 ± 0.63
			$t^2 = 6.62$ [3]	$t^2 = 7.00$ [3]	
Ad libitum	9	10% casein	17.64 ± 1.18	0.41 ± 0.02	64.38 ± 0.57
	9	10% raw pea	9.72 ± 0.47	0.25 ± 0.01	64.44 ± 0.45
			$t^2 = 6.71$ [3]	$t^2 = 8.00$ [3]	

[1] Standard error of single observations.
[2] Calculated from Fisher's formula (Snedecor, '38).
[3] Denotes significance at the 1% level.

Ad libitum feeding did not result in more protein storage per gram of protein fed (0.41 for this group contrasted with 0.39 per gm for those pair-fed, table 4). Since the 2 methods of feeding appear to be comparable, the selection of the one to be used would be a matter of choice. However, if a complete protein such as that of whole egg had been employed in lieu of casein, ad libitum feeding would no doubt have resulted in a significant increase in both protein efficiency and retention.

Biological value expressed either as protein efficiency (table 2) or protein stored per gm eaten (table 4) resulted in about the same difference (approximately 55%) between the casein and raw pea protein.

The biological value of casein at a 10% level determined in a metabolism study proved to be 65.6%, that for peas 57.3%, a difference of 8%. The coefficient of true digestibility for casein from this study was 98.6% and that for peas 91.6%, a difference of 7%. It is doubtful whether the lower digestibility of the pea protein completely explains the lower biological value, as Woods et al. ('43) were able to elevate the protein efficiency of a 10% pea diet above that of casein by the addition of 0.3% methionine.

SUMMARY

1. The protein of raw Alaska field peas is inferior to that of casein when both are fed at the 10% level. Of the pea products studied as sources of protein the germ proved to be slightly but not significantly more efficient, whereas both the sprout and immature pea were significantly lower in value than the raw pea.

2. Supplementation of at least half of the raw pea protein with cereal grains materially improved the biological value presumably because this increased the methionine content.

3. Canning has a very harmful effect on the pea protein probably because the cystine content is lowered by this process. Baking, too, lowers the value significantly.

4. Three methods of determining the biological value of proteins have been compared: protein retention, protein efficiency and a metabolism study. Biological value expressed either as protein efficiency or protein stored per gram eaten resulted in about the same difference (approximately 55%) between the casein or pea protein.

5. The biological value of casein at a 10% level determined in a metabolism study proved to be 65.6%, that for peas, 57.3%, a difference of 8%. The coefficients of true digestibility

for casein and peas from this study were 98.6% and 91.6%, respectively, a difference of 7%.

6. A comparison of 2 groups of animals (pair and ad libitum fed) on a 10% casein diet showed a significant difference in weight gains but not in protein efficiency or protein stored per gram of protein eaten.

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THE EFFECTS OF B VITAMINS, LIVER AND YEAST ON ATABRINE TOXICITY IN THE RAT[1]

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Available data indicate that in addition to the major nutrients, there are substances present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensable under normal conditions, or their requirements are so small that they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to such an extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient (Ershoff, '48). In the present communication data are presented indicating that atabrine is such a stress factor. Administration of this drug in toxic amounts to the young rat resulted in retardation of growth, alopecia, inhibition of ovarian development, granulocytosis and other pathological effects preventable, at least in part, by the administration of an unknown nutrient present in liver and yeast.

[1] The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

PROCEDURE AND RESULTS

Four basal rations were employed in the present experiment: diets A, B, C and D. Diets A and B were purified rations containing the B complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. For the present experiment 64 female rats of the Long-Evans strain were selected at 21 to 23 days of age and with an average weight of 41.8 gm. The animals were kept in metal cages with raised screen bottoms to prevent access to feces, and were fed *ad libitum* the diets listed in table 1. Feeding was continued for 8 weeks or until death, whichever occurred sooner. During the eighth week electrocardiograms were taken of all rats. All animals were autopsied on the fifty-sixth day of feeding, ovarian and submaxillary weights determined, and sections of the ovaries, submaxillary glands, liver and heart prepared for microscopic study. Tissues were fixed in 10% formol and stained with hematoxylin and eosin.

Our findings indicate that the toxic effects of atabrine in the immature rat may be counteracted by dietary means. Pathological effects of atabrine were most pronounced in rats subsisting on the synthetic ration (diet A), and least evident in the animals on the liver-containing diet D. Atabrine-fed rats on diet A exhibited a marked retardation of growth (table 2), extensive alopecia, infantile ovaries and enlarged submaxillary glands (table 2), granulocytosis (table 3) and electrocardiographic abnormalities. Three of the 10 rats on this diet failed to survive the experimental period of 8 weeks; and of the remaining 7 animals only 2 gained weight after the second week of feeding. Eight rats in this series developed extensive alopecia during the first month of feeding although new hair had generally replaced the areas of alopecia by the eighth week. At autopsy ovaries appeared infantile both in weight and microscopic appearance. The most striking finding, however, was a marked hypertrophy of the submaxillary glands. These weighed 3 to 4 times more than those observed in normal

TABLE 1

Composition of experimental diets without (—) and with (+) atabrine.[1]

COMPONENTS OF BASAL MIXTURE	DIETS A_1 and A_2 —	+	DIETS B_1 and B_2 —	+	DIETS C_1 and C_2 —	+	DIETS D_1 and D_2 —	+
Yeast[2]	0.0		0.0		10.0		0.0	
Whole liver powder[3]	0.0		0.0		0.0		10.0	
Casein, "vitamin-test"[4]	22.0		22.0		22.0		22.0	
Sucrose	73.5		73.5		63.5		63.5	
Salt mixture[5]	4.5		4.5		4.5		4.5	

Vitamin supplements

Fat-soluble vitamins: *for all diets,* see footnote 6.

B vitamins: *for diets A, C and D,* to provide levels considerably in excess of minimum requirements, see footnote 7; *for diet B,* see footnote 8.

[1] Atabrine (quinacrine-HCl powder, Winthrop Chemical Co., New York) was incorporated in diets A_2, B_2, C_2 and D_2 at a level of 500 mg per kg of diet, replacing an equal amount of sucrose.

[2] Brewers' type yeast no. 200, Anheuser-Busch, Inc., St. Louis, Mo. Each gram, according to the manufacturer, contained the following vitamin potencies: thiamine 600 μg, riboflavin 75 μg, pyridoxine 100–125 μg, pantothenic acid 420–560 μg and nicotinic acid 350–500 μg.

[3] Whole dried liver powder, Armour and Co., Chicago, Ill.

[4] Vitamin-test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] Salt mixture no. 1 of Sure ('41).

[6] Each rat received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A–D concentrate containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D. The concentrate used was Nopco assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

[7] To each kilo of diets A, C and D were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-1,4-naphthoquinone 5 mg and choline chloride 1.2 mg. The thiamine, riboflavin, pyridoxine, pantothenate and nicotinic acid were administered in amounts comparable to those provided by the brewers' type yeast no. 200 when fed at a level of 12% of the diet.

[8] To each kg of diet B were added thiamine hydrochloride 144 mg, riboflavin 18 mg, pyridoxine hydrochloride 30 mg, calcium pantothenate 134.4 mg, nicotinic acid 120 mg, p-aminobenzoic acid 600 mg, folic acid 10 mg, biotin 1 mg, 2-methyl-1,4-naphthoquinone 10 mg, choline chloride 1.2 gm and inositol 1.2 gm. Doubling the content of these vitamins in diet B (over A, C and D; see footnote 7) provided these factors in amounts comparable to those present in diet C.

animals of similar weight and were significantly heavier than those observed in the much larger atabrine-free controls (table 2). Histologically the increased size of the submaxillary glands appeared to be due almost entirely to hyperplasia and hypertrophy of mucous cells.[2]

Retardation of growth was similarly observed in atabrine-fed rats on diets B, C and D although growth was significantly greater in rats on these rations than in those on diet A. Atabrine-fed rats gained most weight on the liver-containing diet D, with growth somewhat less on diets containing yeast (diet C) or the additional B vitamins (diet B). The growth curves of 3 rats on the latter ration showed a plateau after the first few weeks of feeding; the remainder of the group, however, and all atabrine-fed rats on diets C and D gained weight consistently during the experimental period. With the exception of the 3 rats on diet B whose growth curves showed a plateau, alopecia was not observed in any of the atabrine-fed rats except those on diet A.

The ovaries appeared infantile both in weight and microscopic appearance in all atabrine-fed rats on diet A. They were somewhat larger in atabrine-fed rats on diets B and C but were still significantly smaller than those observed in atabrine-free controls. On diet D no significant difference in ovarian weight was observed between animals fed atabrine and those on similar rations with atabrine omitted; and histologically the ovaries of atabrine-fed rats in this group appeared normal in all respects. On atabrine-free rations ovarian weights did not differ significantly on any of the diets employed; and histologically, ovaries appeared normal in all groups (table 2).

Enlargement of the submaxillary glands was directly correlated with alopecia and failure to gain weight. Enlarged submaxillaries were only observed in atabrine-fed rats that lost fur and whose growth curves showed a plateau (8 rats on diet A; 3 on diet B), with glands apparently normal both in

[2] We are indebted to Professor E. M. Hall, Department of Pathology, University of Southern California Medical School, for examination of the histological material.

size and histological appearance in the remaining animals of the atabrine series. In no instance where atabrine-free rations were used were the submaxillary glands enlarged (table 2).

TABLE 2

Effects of atabrine on growth and ovarian and submaxillary weight in the immature female rat.

DIETARY GROUP	NUMBER OF ANIMALS	INITIAL BODY WEIGHT	GAIN IN BODY WT. OVER 8-WK. PERIOD	AVERAGE OVARIAN WT.[1]	AVERAGE SUBMAXILLARY WT.[1]	RATIO OF SUBMAXILLARY WT. TO BODY WT. $\times 10^{-3}$
		gm	*gm*	*mg*	*mg*	
			Atabrine series			
A	10	41.7	33.9 ± 12.1 (7)[2]	12.4 ± 3.4	486.4 ± 41.6	6.43
B	10	41.9	87.4 ± 10.8 (9)	26.0 ± 3.5	418.6 ± 39.4	3.24
C	10	41.8	33.1 ± 5.1 (10)	30.4 ± 2.7	303.8 ± 10.8	2.15
D	10	41.8	118.2 ± 5.2 (10)	43.5 ± 2.9	317.8 ± 19.4	1.92
			Atabrine-free controls			
A	6	41.7	146.4 ± 9.0 (6)	47.0 ± 2.4	374.8 ± 18.4	1.98
B	6	42.0	152.3 ± 7.8 (6)	49.1 ± 3.0	334.6 ± 16.8	1.72
C	6	41.5	146.8 ± 6.9 (6)	43.2 ± 3.2	317.3 ± 32.8	1.69
D	6	41.7	169.7 ± 9.3 (6)	44.8 ± 2.1	313.5 ± 14.8	1.49

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[2] The values in parentheses indicate the number of animals which survived and on which averages are based.

Both the per cent and the total number of granulocytes per milliliter of blood were markedly increased in atabrine-fed rats fed diet A. During the sixth week of feeding total and differential white cell counts, hemoglobin determinations and total red cell counts were made on the tail blood of all sur-

viving rats. Differential counts were made on smears stained with Wright's stain, 100 cells on each of 2 slides being employed for each analysis. All blood counts were made in duplicate.

On the various diets tested no significant difference in total erythrocytes or hemoglobin levels was observed between animals fed atabrine and those on atabrine-free rations. Erythrocytes averaged 7.3 to 8.1 million per mm^3 of blood for the various groups (range 6.4–9.3), with hemoglobin averaging

TABLE 3

Effects of atabrine on the granulocyte count of the rat.

DIETARY GROUP	NUMBER OF ANIMALS	TOTAL LEUCOCYTE COUNT		GRANULOCYTES	
		Average[1] per mm^3	Range per mm^3	%[1]	Total[1] per mm^3
		Atabrine series			
A	8	17,090 ± 1,540	10,200–24,800	43.9 ± 4.3	7,503 ± 735
B	10	15,900 ± 1,310	8,600–24,100	23.0 ± 1.7	3,657 ± 270
C	10	13,280 ± 1,650	9,200–20,400	24.3 ± 2.5	3,227 ± 332
D	10	12,270 ± 1,280	7,900–18,400	21.5 ± 2.3	2,638 ± 282
		Atabrine-free controls			
A	6	13,150 ± 860	8,800–15,800	18.6 ± 2.6	2,446 ± 342
B	6	12,870 ± 1,040	7,800–18,100	17.4 ± 2.8	2,239 ± 360
C	6	14,180 ± 810	8,200–16,600	19.1 ± 1.6	2,708 ± 227
D	6	13,710 ± 930	10,600–15,600	24.2 ± 2.0	3,318 ± 274

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

15.4 to 16.1 mg/100 ml (range 14.6–17.2). Total leucocytes did not differ significantly for the various rations tested; a significant increase in per cent and total granulocytes was observed, however, in atabrine-fed rats on diet A. Such was not the case with atabrine-fed rats on other diets tested nor for animals on atabrine-free rations (table 3).

In agreement with earlier findings (Hegsted, McKibbin and Stare, '44) no consistent abnormalities were observed histologically in the liver and myocardium of atabrine-fed rats on the various diets employed, nor did these tissues differ sig-

nificantly from those of animals fed similar rations with atabrine omitted.[3] These findings are in contrast to those of Wright and Lillie ('43) and Siegel and Mushett ('44) who observed necrosis and a replacement fibrosis in the liver and myocardium of atabrine-fed rats. These differences may be due, at least in part, to the amount of atabrine fed, the composition of the diets employed or a strain difference in response to atabrine feeding.

Myocardial damage in atabrine-fed rats was indicated, however, by means of electrocardiographic tracings. Electrocardiograms were taken with a Cambridge-Hindle 2 galvanometer electrocardiograph (research type unit) of all surviving rats during the eighth week of feeding. Resistance was standardized for each animal, and the standard 3 leads were taken on unanesthetized rats at a paper speed of 100 cm per second.[4] A marked elevation of the ST segment (indicative of myocardial damage) was observed in the electrocardiographic tracings of 5 of the 7 atabrine-fed rats on diet A, and 5 of the 9 atabrine-fed rats on diet B.[5] Elevated ST segments did not occur in tracings obtained from rats fed diets C_2 or D_2 or from animals fed atabrine-free rations. Further abnormalities in the atabrine series consisted of a prolonged PR interval (indicative of delayed AV conduction) in animals fed diet D_2. Six of the 10 rats on the latter ration had a PR interval of 0.05 seconds or longer in contrast to an approximate value of 0.04 seconds in virtually all rats fed other rations tested. With the exceptions of the above, electrocardiograms of atabrine-fed rats did not differ significantly

[3] See footnote 2, page 272.

[4] We wish to express our sincere appreciation to Dr. John C. Ruddock, for the examination and description of the electrocardiographic tracings. Dr. Ruddock is Clinical Professor of Medicine, University of Southern California Medical School, Chief of Medical Service and Head of the Department of Cardiology, St. Vincent's Hospital, Los Angeles, California.

[5] ST segments were elevated on all 3 leads in rats on diet B_2 and on leads II and III in those on diet A_2. Slight elevations of the ST segment were occasionally noted in leads II and III in animals on all control rations and those on diets C_2 and D_2. In no case, however, were they so pronounced as those in rats on diets A_2 or B_2.

from those of the controls either in complexes or in ventricular rate.[6] No abnormalities were observed in the electrocardiograms of atabrine-fed rats on diet C.

In subsequent work experiments were conducted in an attempt to concentrate the factor or factors in liver responsible for its protective effect. Immature female rats of the Long-Evans strain were weaned at 21 to 23 days of age and fed *ad libitum* the following 3 diets: (1) diet A, (2) diet A plus liver concentrate powder 1–20 [7] added at a level of 4% of the ration, and (3) diet A plus extracted liver residue [7] added at a level of 10%. The liver fractions were added in place of an equal amount of sucrose. All diets were supplemented with 500 mg of atabrine per kilogram of diet. Feeding was continued for 8 weeks (10 animals per group).

The findings indicate that the protective factor or factors is either water-insoluble or chemically bound so that it may not be readily removed by simple water extraction. No significant difference in growth or gross appearance was observed between atabrine-fed rats on diet A and those receiving liver concentrate powder 1–20 (containing the water-extractable material of raw liver). On the other hand, extracted liver residue (consisting of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances) was virtually as effective as whole liver powder in counteracting atabrine toxicity in the rat. Animals fed the above rations made the following gains in weight during the 8-week feeding period: diet A, 41.6 $\pm$ 10.4 gm; diet A plus liver concentrate powder, 52.8 $\pm$ 9.7 gm; diet A plus extracted liver residue, 92.7 $\pm$ 9.3 gm.

DISCUSSION

Available data indicate that factors are present in liver and yeast that will counteract, at least in part, the effects of drug

[6] Heart rates averaged 504 to 537 per minute for all groups with an individual range of 465 to 570 per minute. The QRS interval averaged 0.02 seconds for all groups.

[7] We are indebted to Dr. David Klein of the Wilson Laboratories, Chicago, Ill., for the liver concentrate powder 1–20 and the extracted liver residue employed in the present experiment.

toxicity in the rat. As early as 1922 Funk expressed the view that the composition of the diet and perhaps its vitamin content may have a profound influence on the toxicity of drugs. This suggestion has been amply confirmed, not only in regard to the known nutrients (Ershoff, '48) but to additional factors present in liver or yeast. The beneficial effects of the latter in animals inhaling carbon tetrachloride or fed toxic doses of strychnine, promin, dinitrophenol, sulfanilamide and other drugs have long been recognized (De Santibañez, '43; Battelli, '40; Higgins, '44; Chamelin and Funk, '43). Similar results have been observed following toxic doses of diethylstilbestrol (Funk and Funk, '39; Chamelin and Funk, '43), estrogen (Engel and Rosenberg, '45; Ershoff and Deuel, '46) and desiccated thyroid (Ershoff and Hershberg, '45; Ershoff, '47a, b). The present experiment indicates that atabrine toxicity may also be counteracted, at least in part, by the administration of whole liver or yeast.

Our findings indicate that the effects of atabrine administration in the immature female rat are dependent on the diet employed. With rats fed a synthetic ration (diet A), administration of atabrine at a level of 500 mg per kilogram of diet resulted in marked retardation of growth, alopecia, inhibition of ovarian development, enlarged submaxillary glands, granulocytosis and myocardial damage as indicated by electrocardiographic tracings. The above effects were largely counteracted by the administration of desiccated whole liver at a level of 10% of the diet in place of an equal amount of sucrose. On this latter ration (diet D_2) growth was significantly greater than on diet A_2; alopecia did not occur; ovaries and submaxillary glands appeared normal both in weight and microscopic appearance; granulocyte counts were normal; and electrocardiograms were free of the abnormalities observed on diet A_2 although some prolongation of the PR interval was observed. Similar results were obtained with diet C_2 (in which yeast was fed in place of the whole liver), although growth was less than on diet D_2 and ovaries resembled in weight and microscopic appearance those of an

immature rat. Electrocardiograms in this series, however, remained free of the abnormalities observed in rats on other atabrine-containing rations and were indistinguishable from those of normal controls. Findings for rats on diet B_2 were intermediate between those for animals on diet A_2 and rations containing whole liver and yeast. Three of the 10 rats on this diet failed to grow; they developed alopecia and at autopsy revealed markedly enlarged submaxillary glands. The remainder of the series, however, were indistinguishable grossly or in microscopic appearance from animals fed yeast (diet C_2). Electrocardiographic tracings revealed a marked elevation of the ST segment similar to that observed in 5 out of the 9 rats on diet A_2. On atabrine-free diets no abnormalities were observed on any of the rations employed.

Since the toxic effects of atabrine were less pronounced in rats on diet B_2 than in those on diet A_2, and since these 2 diets differed only in their content of known B vitamins, it is apparent that the beneficial effects of diet B_2 were due to its increased content of B vitamins. The latter ration contained twice the thiamine, riboflavin, pyridoxine, pantothenate and niacin content of diet A as well as significant amounts of biotin, folic acid, inositol and p-aminobenzoic acid. It would appear that one (or more) of the latter factors was responsible for the observed effects. However, the toxic effects of atabrine were still more pronounced on diet B_2 than on yeast or whole liver-containing rations (diet C_2 or D_2). These findings would indicate that in addition to the known B vitamins there were still other factors in the whole liver and yeast that counteracted, at least in part, the effects of atabrine toxicity in the rat. Preliminary results indicate that the protective factor(s) is present in the water-insoluble fraction of liver remaining after the removal of the extractable water-soluble material.

SUMMARY

The administration of toxic doses of atabrine to immature female rats maintained on a synthetic ration resulted in marked retardation of growth, alopecia, inhibition of ovarian

development, enlarged submaxillary glands, granulocytosis and myocardial damage as indicated by electrocardiographic tracings. These effects were largely counteracted by the addition of desiccated whole liver or yeast to the basal ration and, to a lesser extent, by the administration of additional B vitamins. Whole liver was more effective than yeast or the additional B vitamins in promoting growth and ovarian development in the immature atabrine-fed rat. On atabrine-free rations no abnormalities were observed on any of the diets employed. The protective factor(s) was present in the water-insoluble fraction of whole liver. The suggestion is made that in addition to the known B vitamins, still other factors are present in whole liver and yeast that are required in increased amounts by the atabrine-fed rat.

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SELF SELECTION OF DIET

VII. THE EFFECT OF AGE AND PREGNANCY ON SELECTION [1]

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TWO FIGURES

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In a previous experiment, rats 6 to 9 weeks old were allowed their choice of casein, hydrogenated vegetable oil, sucrose, and a salt mixture (Scott, '46). About 40% of the animals refused to eat casein and a majority disliked sucrose as a choice. The present experiments describe the choices of younger (3 to 6 weeks old) and older (12 to 15 weeks old) animals and of animals kept on selection experiments for a long period of time.

Richter and Barelare ('38) reported that increased relative proportions of sodium chloride, olive oil, and casein were selected by animals during pregnancy, and believed these to be associated with increased need for these substances. In the experiments reported here, only an increased relative intake of salt mixture was found during pregnancy.

EXPERIMENTAL

In the first experiment, 15 male and 16 female albino rats were weaned at exactly 21 days of age, and allowed their choice of casein [2] hydrogenated vegetable oil,[3] sucrose, and a

[1] Contribution no. 656 of the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

[2] Labco "vitamin-free."

[3] Primex.

salt mixture (Jones and Foster, '42), each in a separate cup. Vitamins were fed separately as pills.[4] The amount eaten from each cup was determined daily and the cups then interchanged in a predetermined random manner. This procedure was continued for 54 weeks or until the animal died.

In a companion experiment, 10 male and 10 female littermates of the rats used above were weaned at 21 days of age, and given a standard diet [5] plus vitamin pills until 12 weeks old, at which time the average weight of the males was 245 gm and that of the females, 181 gm. They were then allowed the same choices as in the first experiment and the same procedure followed for a 3-week period.

In the pregnancy study, rats from the first experiment were mated when 3 to 10 months old. Twelve successful matings were obtained. Choice of food of the females was recorded in the same manner as above for the 3 weeks of pregnancy and the preceding 3 weeks.

RESULTS

Weanling rats showed a marked lack of ability to select an adequate diet. During the first 3 weeks only 9 rats out of 31 ate enough casein to gain weight, and one of these stopped eating it in the second 3-week period. One rat which ate no casein in the first 3 weeks began eating it in the fourth week. As a result, 22 of the 31 rats in the experiment died of lack of dietary protein at an average period of 37 days after weaning. The choices of the weanling animals during the first 3 weeks are shown in table 1, where they are compared with results on older animals. For tabulation purposes the animals were divided into those that failed and those that succeeded in gaining weight (groups A and B, respectively).

[4] One pill was given each rat daily. It contained approximately: 60 μg thiamine hydrochloride; 120 μg riboflavin; 90 μg pyridoxine hydrochloride; 150 μg calcium pantothenate; 10 mg choline chloride; 1 mg α-tocopherol; and 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola; all in a dextrin-powdered sugar base.

[5] The standard diet consisted of 24% casein, 10% Primex, 62% sucrose, and 4% salt mixture (Jones and Foster, '42).

Three males and 6 females from this group lived for a long period of time. One female died in the thirty-eighth week on selection, after failing to eat protein while pregnant. One male died in the thirty-fifth week, and 1 female died in the fifty-fourth week, both of unknown causes. The average growth and food selections of these 9 animals are shown in figure 1.

TABLE 1

Weight change and food selection of rats at various ages.[1]

	NUMBER OF ANIMALS	CHANGE IN BODY WT.	TOTAL CALORIES EATEN	PER CENT OF TOTAL CALORIES			SALT EATEN
				Fat	Sucrose	Protein	
		gm					*gm*
GROUP A							
3–6 weeks old	22	— 9.3	256	84.7	13.5	1.9	1.2
6–9 weeks old [2]	34	— 21.0	403	76.6	22.3	1.3	0.9
12–15 weeks old	7	— 32.3	1158	73.1	21.6	5.4	1.9
GROUP B							
3–6 weeks old	9	47.1	513	61.2	13.4	25.5	5.1
6–9 weeks old [2]	53	58.9	877	46.7	25.9	27.4	5.4
12–15 weeks old	13	13.9	1264	41.7	32.6	25.8	5.9

[1] Data are 3-week averages.

[2] Results from another experiment (Scott, '46).

Thirteen out of 20 animals put on selection experiments at the age of 12 weeks ate protein and gained weight, while 7 did not. The selections of these animals are also shown in table 1.

With the exception of 1 male, successful matings were obtained with the 9 animals from the first experiment that were used in the pregnancy study. The average size of the 12 litters in the experiment was 8.1, and the young appeared to be of normal size and development. The young of only 1 female showed milk in their stomachs, and in every case the offspring were eaten within a few days. The selections during pregnancy and for the 3 preceding weeks are shown in figure 2. During the last 2 or 3 days before parturition, the mothers ate very little food — about 20 calories per day — and thus the average caloric intake for the final week of pregnancy was lower than that of the preceding week. The high rate of weight gain during the final week appeared then to be due in part to a change in the animals' water balance.

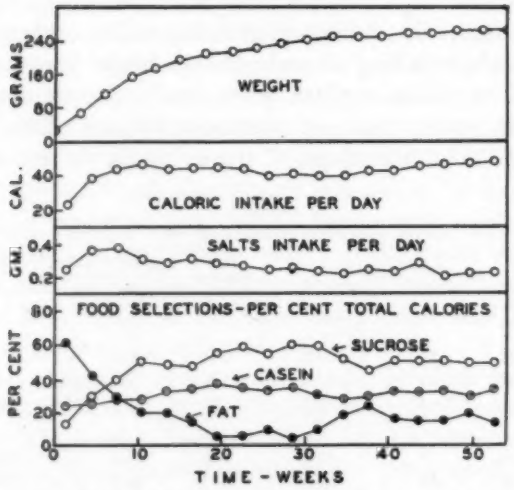

Fig. 1 Average weight and food selections of 9 rats over a long period. Each point represents an average for a 3-week period.

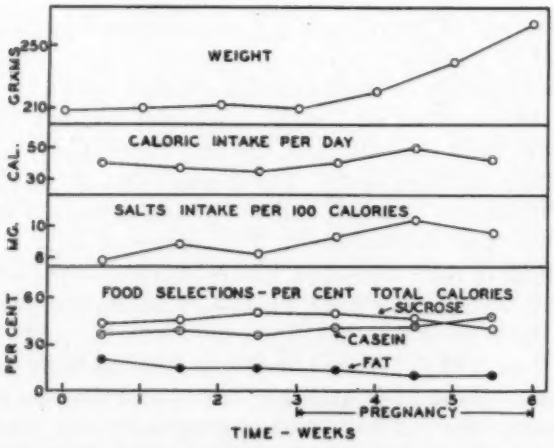

Fig. 2 Average weight and food selections before and during pregnancy. Each point represents a weekly average.

DISCUSSION

The most obvious change associated with age in the choices of young rats was in the appetite for casein. The probability that as few as 9 out of a random sample of 31 animals would select casein was less than 1 in 1000, if the calculated probability of a rat selecting protein was 0.61. (The probability of an animal eating casein as determined with 6-week old animals was 0.61 [Scott, '46]). On the other hand, almost the same proportion of 12-week old animals (13 out of 20) selected casein, as was found with 6-week old animals (53 out of 87).

Another change in appetite with age was in the relative preference of young rats for fat and their relative avoidance of sucrose. Older animals had a greater preference for sugar and less for fat. In the long-term experiment, the only marked change in appetite was likewise a substitution of sugar for fat with advancing age. Neither of these 2 changes with age — protein appetite, and sugar versus fat appetite — appeared to have an obvious explanation.

The growth rate of the animals in the long-term experiment is of some interest, since, even disregarding the many animals which never grew, the rate is inferior to that of animals on a standard diet. At 12 weeks of age, 3 male rats on self-selected diets averaged 138 gm in weight, while 10 males on standard diet (12 weeks' experiment) averaged 245 gm. Similarly, 6 females on self selection averaged 167 gm, while 10 females on standard diet averaged 181 gm in weight. This is not in agreement with an earlier report (Richter, Holt and Barelare, '38).

During pregnancy, the food intake of the females increased, but the proportions of fat, sugar and casein eaten were almost constant. The intake of salts did increase relative to the caloric intake, however.

SUMMARY

Only 9 out of 31 animals 21 days old selected casein and exhibited some growth when offered a choice of that protein,

sucrose, hydrogenated vegetable oil, and salts. The other 22 died at an average age of 58 days. About 60% of either 6-week old or 12-week old animals selected casein and gained weight. Younger animals showed more preference for fat and less preference for sucrose than older rats. During pregnancy, the intake of salts increased.

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